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Biophotonics in Stem Cell Research and
Developmental Biology

Nizhny Novgorod, Russia
21 – 27 July, 2013

Russian Academy of Sciences
Institute of Applied Physics

IV International Symposium
**TOPICAL PROBLEMS
OF BIOPHOTONICS**



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Nizhny Novgorod, Russia

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Plenary Talks

THERANOSTICS: MULTIFUNCTIONAL AGENTS FOR DIAGNOSTICS AND THERAPY

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Abstract. Rapid development of nanotechnology has stimulated considerable interest in their applications in life sciences. In particular, several unique features of nanoparticles appeared very attractive for their implementation as diagnostic and therapeutic complexes. An important attribute of the nanoparticles is the ability to bind various molecules ensuring biological activity of the particles; among these molecules are toxic and/or visualizing modules, as well as targeting agents for addressed delivery. The recent results on bioimaging applications of barnase-barstar platform with important types of the nanoparticles, including quantum dots, luminescent nanodiamonds, colloidal gold, magnetic and luminescent upconversion nanoparticles are reviewed.

Nowadays nanobiotechnologies open up new possibilities for diagnostics and treatment of oncological, cardiovascular, autoimmune, and other diseases. Nanoparticles (NPs) attract a particular attention as new and unique therapeutic agents that make it possible to solve old but still actual problems by principally new means and ways. A number of nanoparticle-based medications have already been approved for therapeutic purposes. Important advantage of NPs is their developed surface, which can be decorated with biocompatible functional moieties, and thus form a versatile docking station. NP can serve as a nano-vehicle to host biologically significant modules, such as therapeutic, targeting and stealth modules for targeted delivery, diagnosis that guides and monitors effects of the NP-assisted therapy of pathology lesions. These properties provide foundations for significant emerging areas in applied biomedical science including (personalised) nanomedicine and theranostics.

In order to apply nanoparticles for imaging and/or therapy one needs to consider three key aspects: design of bright and photostable luminescent nanomaterials conspicuous on the background of the excitation light back-scattering and cell autofluorescence; amiable surface modification to enable facile interfacing with biomolecules, and modular engineering of the biomolecular complexes with targeting vectors (antibody, mini-antibodies or peptides) firmly attached to the NP for target delivery to specific cellular or tissue sites.

To develop a modular engineering concept (Fig. 1) we study self-assembly of polystyrene micro- and nanoparticles with two functionalities – magnetic and fluorescent – using proteinaceous ‘molecular glues’, most notably, the barnase–barstar system (BBS). The obtained assemblies were tested for their resistance to high concentrations of chaotropic agents (urea and GdmHCl) as well as high temperature and low pH conditions causing denaturation of most proteins. In the majority of cases, the structures exhibit unusual stability and maintain apparently unaltered morphologies upon exposure to these conditions for extended periods of time. Comparison of the BBS-system with other proteinaceous self-assembly systems (streptavidin_biotin, antibody_antigen, and protein A_immunoglobulin), showed that whereas their resistance to destruction is relatively comparable, the capacity to assemble under harsh conditions differs substantially.

The ability of the BBS-glued assemblies to retain their integrity in extreme conditions makes them attractive for a number of applications, taking into account the feasibility of utilization of modules of various nature as participants of self-assembly. The designed nanoparticle assembly approach may prove particularly advantageous for such applications where the remarkable durability of the assemblies becomes a feature of high value. Examples embrace a broad spectrum including sensing of ecological pollutants in complex media, photonics and theragnostic approaches in medicine, also making use of multifunctionality offered by the assemblies.

Furthermore, the unexpectedly high ‘tensile strength’ of the proteinaceous molecular glues described in this work sets one thinking of potential applicability of these self-assembled structures instead of, or alongside with, covalently linked entities. If for creation of *a fortiori* very durable and ‘reliable’ structures at the nano- and microscale one would definitely choose chemical reactions as a

means to build such structures, now, armed with the knowledge of exceptional stability of protein-assisted assemblies, one has access to a great variety of specific (naturally occurring or engineered) ‘molecular glues’ that can be used for the same purposes and with similar efficiency. Moreover, utilization of specific non-covalent interactions adds to the flexibility of the designed assembly systems and imparts higher controllability over the whole process of assembly than in the case of chaotic chemical reactions.

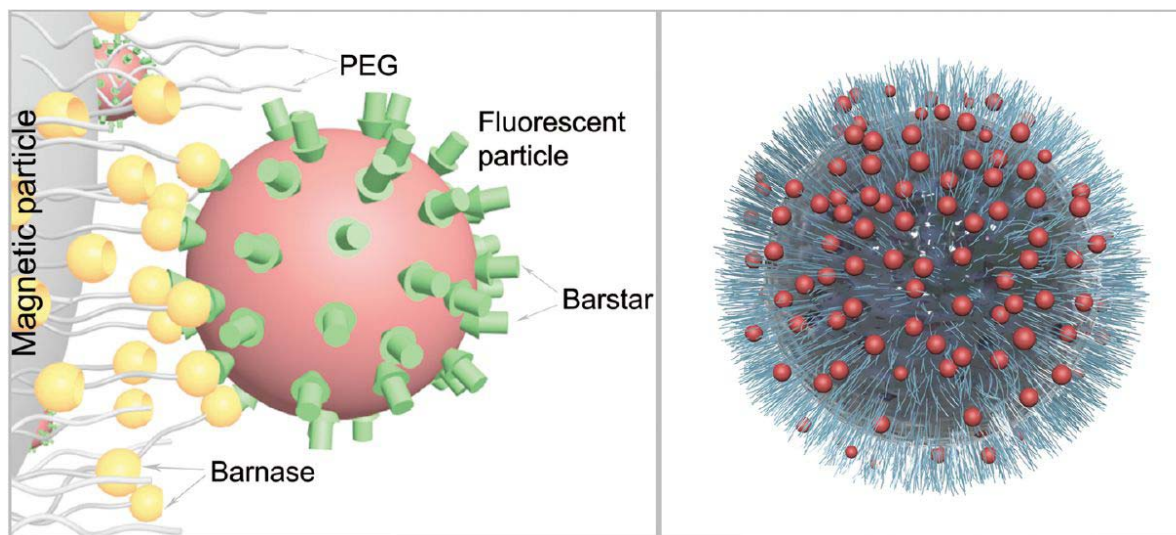


Fig. 1. The concept of multipoint contacts between the components of the colloidal assembly. Left: The multiple BBS pairs at the particle interface. Right: A general schematic view of an assembled structure. PEG, poly(ethylene glycol)

In the paper we also review our recent results on bioimaging applications of BBS with important types of the nanoparticles, including well established quantum dots (QDs), luminescent nanodiamonds (LNDs), colloidal gold, magnetic NPs, and luminescent upconversion NPs.

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MULTI-PHOTON NANOSCOPY AND SUPER RESOLUTION MICROSCOPY

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Abstract. Non-linear excitation of fluorescence as implemented in multi-photon microscopy coupled with far-field optical nanoscopy and super resolution methods have a great impact in medicine, biology, bioengineering and biophysics. Such a merging allows designing new experiments towards applications related to thick three-dimensional specimens. Moreover, non-linear excitation allows integration with other mechanisms of contrast like second harmonic generation, providing an important label free set of complementary information. Here we review some recent implementations of far-field super resolution methods taking advantage of two-photon excitation/photoactivation processes.

Multi-photon excitation (MPE) microscopy and its two-photon excitation (2PE) implementation [1] are key methods for answering questions in biological sciences and medicine. More specifically, 2PE is particularly well suited for studying living cell aggregates or tissues up to a significative depth, today close to one millimeter. Further advantages of two-photon excitation include: localized volume of excitation and emission, background rejection especially in 3D imaging, intrinsic optical sectioning, 3D localized photobleaching, extremely localized photoswitching and uncaging, long term experiments [2]. These features have made possible experiments and innovations beyond the capability of traditional confocal microscopy. Unfortunately, one of the practical drawbacks in 2PE fluorescence microscopy is given by the worsening of spatial resolution due utilization of red shifted wavelengths with respect to 1PE. Image restoration [3] or aperture engineering [4] approaches have been used to alleviate such a condition. In 1994, Hell and Wichmann proposed the stimulated emission depletion (STED) concept as a way to improve resolution in optical fluorescence far-field microscopy [5]. This is made in practice circumventing the diffraction barrier by precluding simultaneous emission of adjacent (distances shorter than the one imposed by the Abbe's law) spectrally identical molecules. This approach has been also implemented under 2PE conditions since it affects molecules in the excited state [6].

Considering the advantages related to 3D-2PE fluorescence microscopy [6], a special 2PE-STED architecture, to enhance spatial resolution performances, was implemented [7]. Since one of the main goals, in medical and biological applications, is to address questions related to thick highly scattering specimens, a SW (single wavelength)-2PE-STED approach has been designed, implemented and demonstrated [7]. A very same wavelength is used to prime 2PE delivered at high peak intensity and short pulsewidth (100 fs) and to perform fluorescence depletion at larger pulsewidth (200 ps) that inhibit the 2PE possibility. Demonstration that a widely used red-emitting fluorophore, ATTO647N, can be two-photon excited at a wavelength allowing both 2PE and STED using the very same laser source, will be discussed. This fact allows performing 2PE microscopy at four to five times STED-improved resolution, while exploiting the intrinsic advantages of 2PE.

In 2006 precise localization of single molecules at the nanoscale have been implemented on the stream of a seminal PALM approach [8]. Such methods utilize the photophysical properties of fluorescent molecules by enabling the detection of sparse individual molecule photo-switched signatures mainly on thin samples or single cells. As a first step to 3D imaging in thick scattering cellular aggregates we implemented an individual molecule localization (IML) approach within a selective plane illumination microscopy (SPIM) set-up [9]. IML-SPIM allowed us to achieve 3D super resolution in tumoral mammalian cell spheroids [10]. In order to improve the penetration depth in highly scattering samples a 2PP (two photon photoactivation) IML-SPIM approach has been addressed [11] following 2PE-SPIM demonstration [12]. The effects induced by light-sample interactions and a comparison between the performances of 1PE and 2PE in scattering samples have been analyzed quantitatively [11]. Moreover nanoscale lithography has been addressed using a triplet state absorption mechanism realized in a STED-like architecture geometry [13, 14].

Such technological advances are expected to contribute to further growth of and access to multimodal super resolution imaging of 3D biological systems. An incredibly powerful battery of

super resolution tools is available. Combining the features of different imaging methods and modalities enables exploring previously unseen aspects of biological systems at the nanoscale.

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PHOTODYNAMIC THERAPY: A SLICE OF CLINICAL BIOPHOTONICS BRIDGING SCIENCE, TECHNOLOGY AND MEDICINE

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Abstract. Photodynamic therapy (PDT) is a promising treatment modality that involves excitation of a photosensitizer (PS) by photons of a specific wavelength to produce reactive species that are toxic to cells. As the complexity of the target disease increases for reasons such as heterogeneity in disease biology or diffuse disease spread over large surface areas, so must the sophistication in PDT planning and application. This presentation will focus on the challenges associated with applying PDT for treatment of complex disease. Intricacies associated with formulation and delivery of targeted PS, dosimetry, and combination therapies that address cancer-sustaining pathways will be discussed.

Light-activated molecules dissipate absorbed energy via several pathways. Most relevant to this presentation are the photochemical and the fluorescence modes of energy dissipation. Photochemical processes have proved to be useful in the study of biological mechanisms and in therapeutic strategies. The best known application of photochemistry with exogenous chromophores to treat disease is Photodynamic Therapy (PDT), which involves the activation of certain molecules called photosensitizers (PS) with light of a specific wavelength [1, 2]. The general schematics relevant to PDT are presented in Fig. 1. Upon absorption of a single quantum of light, the PS is typically excited to its first excited singlet state from which it can fluoresce by relaxing to the ground state directly. Alternatively, there is intersystem crossing to a triplet state, which is relatively long-lived and capable of photochemistry which leads to the formation of activated molecular species such as free radicals. The triplet state can also exchange collisional energy with ground state oxygen to produce singlet oxygen, the toxic state of oxygen.

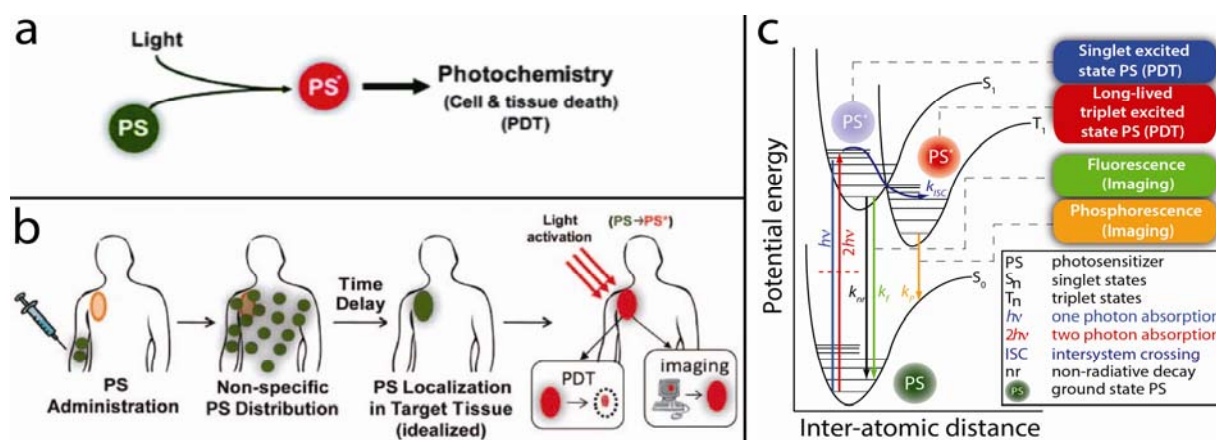


Fig. 1. PDT-mechanisms and clinical application. Light of a specific wavelength excites a PS, yielding photochemistry that results in cytotoxicity (a). Idealized schematic depicting PDT clinical workflow (b). Jablonski diagram showing the quantum mechanical interactions that are typical of excited PS (c). Portions of this figure were taken from reference 1 (Celli et. al., *Chem Rev*, 2010)

Most sensitizers in clinical use exhibit finite quantum yields for both fluorescence and the photochemical process thus allowing for the same molecule to be used for diagnostics and for therapeutics. These PS may, therefore, be viewed as theranostic agents. Regulatory authorities worldwide have approved PDT for several indications and PDT agents are used as fluorescent markers for surgical guidance [1, 3]. Development of new agents and applications to new indications continues.

The advantage of the PDT approach is the potential for dual selectivity: first, selectivity arises from the preferential accumulation of the PS in tumors and second, from the confinement of light to defined volumes. With the advent of portable lasers and sophisticated optical fibers, delivery of light to remote anatomical sites is possible, although appropriate dosimetry remains a challenge.

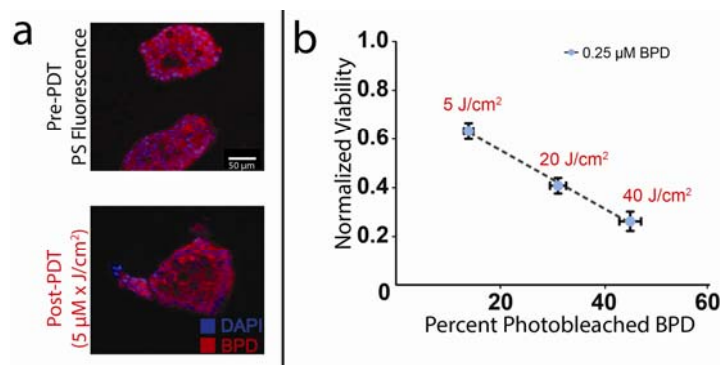


Fig. 2. Example of preclinical PDT dosimetry: three-dimensional model of epithelial ovarian cancer (EOC). Cryosectioned 3D EOC nodule exhibiting benzoporphyrin-derivative (BPD) and DAPI fluorescence imaged before and after PDT (a). PDT treated tumors exhibit on average less PS fluorescence than untreated tumors. Correlation between the normalized viability of PDT treated EOC nodules and BPD photobleaching (b). Portions of this figure were taken from reference 5 (Rizvi et. al., *Phtchm&PhtBiol*, 2013)

Complexity arising from the interaction of different parameters that govern PDT efficacy, including PS uptake at the target tissue, light delivery parameters, oxygen concentration etc., contributes to variability in PDT outcomes observed in patients. PDT dosimetry attempts to diminish this variability by measuring a treatment related parameters that provides predictive insight into efficacy and deposited dose. Several approaches to PDT dosimetry have been investigated over the last few decades [4]. One of which entails quantification of PS photobleaching by measuring PS fluorescence either discretely or continuously during light irradiation. For example, a recent study demonstrated that PS photobleaching correlated well with treatment efficacy in 3D ovarian cancer nodules following BPD-PDT (Fig. 2) [5]. Similar observations have been consistently reported in more complicated animal models [6, 7], and a recent clinical study demonstrated that PpIX photobleaching was a good reporter of PDT induced erythema and edema in human skin (data not shown).

For the application of PDT regionally where the tumor is not necessarily localized but somewhat diffuse, there is need for higher selectivity of delivery of the PS. Because cancer is a complex and heterogeneous disease, there is general consensus that best therapeutic results will be achieved when several cancer-sustaining pathways are destroyed simultaneously. The outcome is further enhanced if the combinations synergize with each other [8, 9]. In this context, we have developed many constructs that are capable of depositing more than a single therapeutic agent. This allows for combination therapies where the combination agents are delivered simultaneously. Examples of such constructs are protein conjugates, polymer-associated agents or liposomally encapsulated molecules [10, 11].

Taking advantage of the dual property of photochemistry and fluorescence of most PS, another focus of our group has been the use of imaging to guide therapeutics. For example, in a study that showed that while PDT killed a majority of cancer cells in an orthotopic prostate cancer model, there was increased secretion of a protein, vascular endothelial growth factor (VEGF) that could compromise the long-term benefit of the treatment [12-15]. This increase in VEGF is seen with many treatments and is probably responsible for incomplete therapeutic outcome by supporting recurrence and metastasis. An imaging strategy was developed which allowed us to monitor in real time the production of VEGF, which in turn allowed us to design the optimal timing of the addition of the anti-VEGF agent and demonstrate a more effective control of tumor burden [16].

In summary, PDT is advancing as part of the overall arsenal for treatment of cancer and non-cancer diseases. The recognition that the same molecules can be used for imaging and therapy enhances the application of the technology although much remains to be accomplished to achieve the full potential of PDT.

Acknowledgements

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APPLICATION OF TWO-PHOTON FRET-FLIM IN STUDY OF CYTOSKELETAL DYNAMICS DURING STRUCTURAL PLASTICITY OF DENDRITIC SPINE

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Abstract. Synaptic cytoskeleton is an essential mechanism for synaptic plasticity. However, because of the size of the synapse, it has been difficult to visualize the exact dynamics of cytoskeletal proteins within synapse during synaptic plasticity. We therefore, employed FRET-FLIM combined with two-photon laser scanning microscopy and photouncaging of glutamate to visualize dynamics of actin cytoskeletal components within dendritic spine. As a result, we could successfully visualize time-lapse of specific interaction of actin and actin binding proteins that supports structural plasticity of dendritic spine.

Introduction

Most excitatory synapses in the mammalian brain are located on dendritic spines, tiny protrusions arising from the dendrite that act as chemically and electrically isolated micro-compartments. Spines exhibit various forms of structural and functional plasticity. In response to the specific modulation of input activity, the strength of the synaptic transmission can be either potentiated (as in Long Term Potentiation - LTP) or depressed (as in Long Term Depression - LTD). Simultaneously, spines can undergo structural changes, enlarging during LTP and shrinking during LTD. In the CA1 region of the hippocampus, LTP is initiated by the entry of Ca^{2+} through NMDA-type glutamate receptors (NMDAR), which triggers the translocation of specific proteins to the synapse, including AMPA-type glutamate receptors (AMPA).

The underlying mechanism of such activity-dependent reorganization is not well understood. We were interested in the dynamics of actin since actin is a ubiquitous postsynaptic protein which can serve both as a structural framework and as a scaffold for other proteins. It can be dynamically regulated by various signal transduction machineries including Ca^{2+} , small GTP-binding proteins, and various other actin binding/regulating proteins. However, it has not been totally clear how actin is regulated during synaptic plasticity. This is mostly because the size of dendritic spine as well as the diameter of actin filament are too small to be visualized with optical microscopy. This hampered the detailed analysis of actin within dendritic spine. We therefore, decided to develop a FRET-based technique to visualize dynamic interaction of actin and its interacting proteins. We employed FRET-FLIM combined with two-photon laser scanning microscopy and photouncaging of glutamate to visualize dynamics of actin cytoskeletal components within dendritic spine.

Methods

Animal experiments were conducted according to the MIT and RIKEN Committee for Animal Careguidelines. Hippocampal organotypic slice cultures were prepared from postnatal day 6-7 rats as described⁸. Slices were cultured at 35°C on interface membranes (Millipore) and fed with MEM media containing 20% horse serum, 27mMD-glucose, 6mM NaHCO_3 , 2mM CaCl_2 , 2mM MgSO_4 , 30mM HEPES, 0.01 % ascorbic acid and 1 $\mu\text{g/ml}$ insulin. pH was adjusted to 7.3 and osmolality to 300-320 mOsm. Slices were biolistically transfected (BioRad) after 6 days in vitro (DIV).

Time-lapse fluorescence imaging was carried out with a modified 2P microscope (FluoView FV1000MPE, Olympus) equipped with two mode-locked femtosecond-pulse Ti:sapphire lasers (MaiTai HP, Spectra-Physics). Slices were maintained in a continuous perfusion of artificial cerebrospinal fluid (ACSF) containing 119 mMNaCl, 2.5 mMKCl, 3 mM CaCl_2 , 26.2 mM NaHCO_3 , 1 mM NaH_2PO_4 and 11 mM glucose, 1 μM tetrodotoxin, 50 μM picrotoxin and 6 mM 4-methoxy-7-nitroindolyl (MNI)-L-glutamate (Tocris, Bristol, UK) equilibrated with 5% CO_2 /95% O_2 . Imaging was performed at 8-9 DIV in primary or secondary dendrites from the distal part of the main apical dendrite of CA1 pyramidal neurons. We induced structural LTP on thin or small mushroom spines, with clear head and neck. 2P uncaging of MNI-glutamate was performed at 720 nm and green and red fluorescence images were simultaneously taken at 910 nm. sLTP was induced by 1 ms pulses repeated at 1 Hz for 1 min aiming close to the tip of the spine. Laser intensity (3-5 mW) was adjusted for each

experiment to obtain a stable spine volume increase of 50-100% for at least 30 min. FLIM was recorded with SPC-730 or SPC-830 (Becker & Hickl) with GdAsP detector H7422-40 (Hamamatsu Photonics). The detection was synchronized with excitation laser by sampling a small part of a two-photon laser. The lifetime was calculated with a custom written software on Igor Pro (Wavemetrics).

Results and Discussion

By using FRET between actin monomers, we found that the actin polymerization-depolymerization equilibrium is modulated rapidly in response to a wide range of frequencies of synaptic activity, in accordance with potentiation or depotentiation of synaptic transmission dependent on NMDA receptor activation. LTP-inducing tetanic stimulation protocol moved the equilibrium towards polymerization, while long-term depression (LTD)-inducing low-frequency stimulation induced depolymerization. The change persists notably for at least 30 minutes after the stimulation, and is accompanied by either structural enlargement (LTP) or shrinkage (LTD) of dendritic spines.

We then analyzed the time-lapse of the postsynaptic protein composition during the selective potentiation of individual spines. We found that multiple proteins were delivered to the synapse in four distinct dynamic patterns and in three sequential temporal phases. Among them, we found that cofilin showed a unique behavior: it showed a rapid and persistent accumulation at the base of spine head. By FRET analysis, we found that accumulation of cofilin is induced by a direct interaction between cofilin and actin and that the density of cofilin at the spine head is high so that it may stabilize the F-actin. We speculate that this mechanism regulates spine expansion and propose a broad mechanistic model for spine reorganization after LTP induction, which explains a number of features associated with synaptic plasticity and metaplasticity and suggests a molecular mechanism for the process of synaptic tagging and capture.

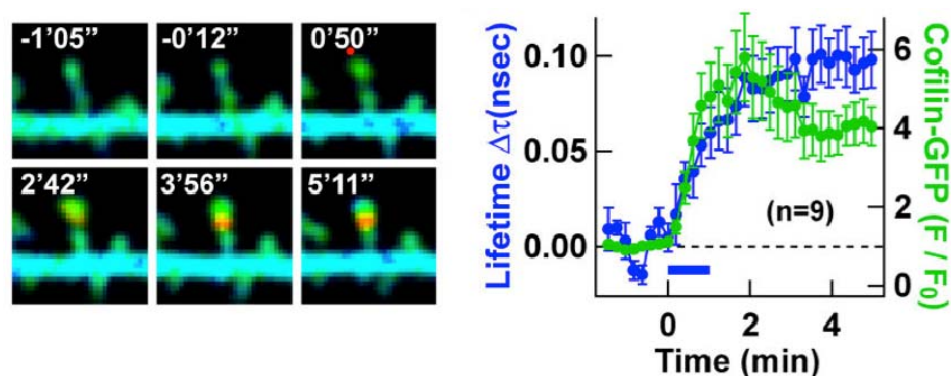


Fig. 1. Interaction between cofilin and actin visualized by FRET-FLIM. Warmer hue indicates higher FRET

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HIGH AND SUPERRESOLUTION MICROSCOPY FOR PROBING PROTEIN-PROTEIN INTERACTIONS AND SUBCELLULAR DYNAMICS

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Abstract. Superresolution beyond the diffraction limit by e.g. photoactivation localization microscopy (PALM) has become indispensable for studying subcellular processes and structures in the nanometer range. The output is a spatial point pattern of molecular positions of a given labeled protein ensemble. These point patterns contain also information about spatial correlations like clustering. Clustering is characterized by the deviation from the arrangement of independent random events. Thus, deviations provide information about the underlying molecular interactions. Here we show that spatial statistics can be utilized to determine the stoichiometry of macromolecular assemblies in cell membranes of neuronal and non-neuronal cells.

Probing non-covalent protein-protein interactions on the nm scale in cell membranes using TIRF-PALM

Structural quantification of molecular ensembles on a nanometer scale is particularly important as the functional role of biological macromolecules is greatly influenced by the microstructure. We see a need for a method quantifying the exact stoichiometry of macromolecular assemblies in cell membranes. Optical and electron microscopy, can be employed to determine information with nanometer resolution. While imaging of continuous biological structures is diffraction limited in far field microscopy due to the overlap of the point spread functions (PSFs) of single fluorescent emitters, superresolution beyond the diffraction limit by photoactivation localization microscopy (PALM) [1, 2] and stochastic optical reconstruction microscopy (STORM) [3, 4] have therefore become indispensable for studying biological processes. By using photoactivatable or photoswitchable labels they keep the concentration of emitters low which allows for the nm precise localization of a diffraction limited spot. Both techniques therefore use the nm precise localization of a diffraction limited spot which is in general given by an Airy function. Near the detected peak it can be well approximated by a gaussian function because recorded fluorescence images are rarely precise enough to distinguish between these two functions. Although the details within the diffraction-limited spot cannot be resolved its location can be determined with a very high accuracy by finding the center of the spot. Currently, this is done by state of the art least squares iterative Gaussian fitting. The output of these techniques is a spatial point pattern of molecular positions of a given labeled ensemble. However little is known about the underlying relations between measured finite point patterns and distribution of biological macromolecules like proteins. Here we examined various approaches to view the measured data in terms of point processes. The statistical properties of these point processes reflect the spatial distribution of the molecular ensembles and can therefore provide considerable information about the system.

PALM offers the powerful possibility to study the distribution of single labeled proteins in the nm range. The output of this technique is a spatial point pattern of molecular positions of a given labeled protein ensemble. These point patterns, however, should contain information about spatial correlations like clustering. Clustering is characterised by the deviation of the pattern from the arrangement of independent random events. The random distribution is given by a Poisson process. Thus, deviations, i.e. the degree and spatial scale of clustering or repulsion in such patterns provide information about the underlying molecular interactions. We explored whether spatial statistics based on k-Nearest Neighbour Analysis in comparison to Ripley's K- or L- function [5] or pair correlation analysis [6,7] can be utilized to determine the stoichiometry of macromolecular assemblies in cell membranes. k-Nearest Neighbour Analysis seems to be a very helpful tool to study the different signal contributions in detail.

Macromolecular assemblies are often found as homomers in living cells. As simple models for proteins with strong non-covalent-interactions we expressed fusion constructs of the Shaker-IR potassium channel composed of four subunits and the selective, cation-permeable, ligand-gated acid-sensing hASIC1a ion channel, which is known to form trimers in the plasma membrane of HEK293

cells. Using quantitative analysis of TIRF-PALM image stacks we succeeded in counting the exact number of monomers in these membrane protein complexes. Comparison with simulations of point patterns of different clustering degrees and of differing localization precision corroborated our findings.

Probing protein-protein interactions during synaptic vesicle recycling in neurons

Fusion of synaptic vesicles (SVs) during fast synaptic transmission is mediated by SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex assembly formed by coil-coiling of three members of the protein family: synaptobrevin 2 (Syb2) and the presynaptic membrane SNAREs syntaxin-1A and SNAP-25. In order to maintain neurotransmission exocytosed SV components need to be retrieved from the surface by compensatory endocytosis. Clathrin-mediated endocytosis (CME) is thought to be the predominant mechanism of SV recycling. However, it might be too slow for fast SV recycling. Therefore, it was suggested that a pre-sorted and pre-assembled pool of SV proteins on the presynaptic membrane might support a first wave of fast CME. We monitored the temporal dynamics of such a ‘readily retrievable pool’ of SV proteins in hippocampal neurons using a novel probe, CypHer 5, a new cyanine dye-based pH-sensitive exogenous marker, coupled to antibodies against luminal domains of SV proteins. This way we could demonstrate the preferential recruitment of a surface pool of SV proteins upon stimulated endocytosis [8].

More recently, using fluorescence nanoscopy (isoSTED, FPALM, and STORM) of labeled SV proteins we could resolve the spatial distribution of the surface pool at the periaxial zone and identify early steps of its formation. Using quantitative k-nearest neighbour and paired correlation analysis of TIRF-PALM image stacks from surface pool constituents we could identify dimerisation of the vesicular SNARE Syb2 as a first important step in self-assembly of these surface nanodomains. In a second step the second most abundant SV protein synaptophysin (Syp) seems to coordinate and sequester these dimers in larger nanodomains or clusters containing a few ten molecules of both Syb2 and Syp – this way efficiently clearing release sites from freshly added SV constituents and preventing cis-SNARE complex formation, which would otherwise result in short-term depression [9]. This finding was corroborated in Syp knockout mice, where cultured hippocampal neurons show defects in the sorting and clearance of Syb2 from the SV release sites when stimulated at higher frequencies. Our results clearly demonstrate the pivotal functional role of syp-syb2 interactions to form clusters of SV proteins at the surface for SV regeneration at the synapse.

In summary, we show that quantitative k-nearest neighbour of TIRF-PALM image stacks can be used to faithfully extract information about protein-protein interactions in functional membrane domains on the nm scale.

Acknowledgements

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LABEL FREE MICROVASCULAR IMAGING OF HEALTHY AND DISEASED WITH DOPPLER OPTICAL COHERENCE TOMOGRAPHY

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Abstract. We present results of imaging human skin microvasculature as well as retinal microvasculature with high speed swept source Doppler OCT (DOCT). For skin imaging, the imaging penetration depth was enhanced by employing Bessel beams that exhibit favorable properties for vascular imaging. For imaging retinal vasculature, high speed laser technology has been employed in combination with an intensity difference technique for vascular contrast. Especially for retinal imaging the advantage of DOCT of being label free and contact free opens new perspectives for early diagnosis of major retinal diseases. We present examples for both healthy tissue vasculature as well as characteristic examples of vascular alterations in diseased tissue.

Extended Summary

Information about tissue perfusion and vascular structure is certainly most important for assessment of tissue or personal health, and the diagnosis of any pathologic conditions. It is therefore of key medical interest to have tools available for both quantitative blood flow assessment as well as qualitative vascular imaging. High-speed data recording maintaining high image quality offered large flexibility, which resulted in novel signal processing schemes, supported by dedicated scanning patterns. We have today a rich variety of DOCT methods available for both quantitative perfusion assessment, as well as for flow contrasting down to the level of individual capillaries [1]. In the following we use an intensity difference technique for vascular contrasting. An angiographic image volume $P(x, y, z)$, contrasting flow against static tissue, is obtained by calculating the squared intensity difference between successive tomograms [2]:

$$P(x, y_i, z) = (I(x, y_i, z) - I(x, y_{i+1}, z))^2,$$

where $I(x, y, z) = 20 \cdot \log \left[\left| \text{FFT}(I(x, y, k)) \right| \right]$, (x, y, z) are the spatial pixel coordinates corresponding to fast and slow scanning and depth coordinate, respectively, and k is the wavenumber. Such method requires good correlation for static tissue over successive tomograms. This is obtained by driving the slow axis scanner with a multiple-step function. It allows measuring N tomograms at the same position y .

Assessment of the retinal and choroidal vascularization is of important diagnostic benefit for major ocular diseases that affect the vascular network already at an early state. The visualization of the microvasculature yields an easy accessible and intuitive way to assess its integrity. The gold standards for their visualization are fluorescein angiography (FA) and indocyanine green angiography. The invasiveness of these techniques together with undesirable side effects, through the injection of a fluorescent dye, limits the screening capabilities for large populations. Therefore DOCT angiography is an attractive alternative as it is non-invasive, label-free, and easy to operate. The availability of both intensity information and vascular contrast with the same OCT data set might soon establish this technique for patient screening, as well as for treatment monitoring. The development of ultrahigh speed OCT techniques based on Fourier domain mode locked (FDML) lasers for SSOCT allowed for A-scan rates beyond 1 MHz [3]. Recent results showed that ultrahigh speed is a prerequisite for flexible and comprehensive vascular contrast imaging with DOCT over a large field of view (FOV). Ultrahigh-speed FDOCT is therefore a promising candidate to compete with the FOV and resolution of fluorescein angiography, since a large patch can be covered by a single recording in a few seconds. Angiography using intensity variance analysis is especially suitable for high-speed SS based system, because it is independent of any acquisition timing problems such as trigger jitter. Also the fact that it does not require bulk motion correction alleviates the post-processing effort as compared to other techniques. The setup for SSOCT with dual balanced detection is found in [2].

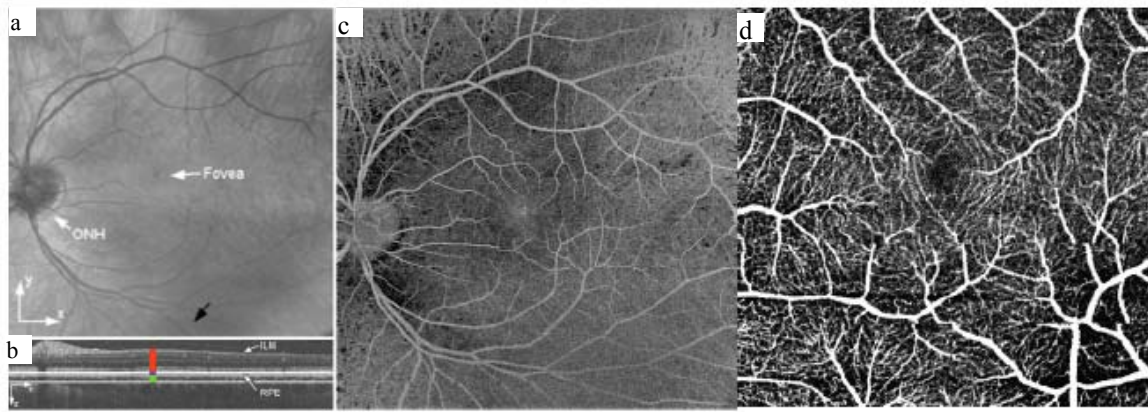


Fig. 1. (a) Pseudo-SLO fundus by en-face mean projection of the intensity data set. ONH: optic nerve head. (b) 5 fold averaged tomogram after flattening to the retinal pigment epithelium (RPE). (c) 48° widefield angiogram, en-face mean projection of the intensity variance 3D data set calculated from (a). (d) ~12° FOV centered at the fovea showing retinal vasculature

Figure 1 shows results of retinal vasculature for a healthy volunteer. Even smallest capillaries can be resolved in the foveal region. We will further present cases of age-related macula degeneration with visible vascular changes including neovascular growth.

The application of OCT to dermatology is generating raising interest. Several studies have already been conducted to investigate inflammatory skin diseases, such as dermatitis, or skin cancer, such as basal cell carcinoma [4]. They were, however, limited to analysis of structural changes assessed from OCT intensity pictures alone. The capability of OCT to visualize also the vascular network has the capacity to provide complementary information related to the important metabolic tissue demand. For skin imaging an extended focus system employing Bessel beams has been built. In the following we demonstrate label free angiography of skin using an extended focus OCT (xf-OCT) system [5]. xf-OCT that is based on Bessel beam illumination provides an enhanced depth of focus as compared to standard Gaussian illumination. This system operates further at a center wavelength of 1300nm for optimal penetration into skin tissue [6]. It is based on swept source OCT and provides high-speed imaging by employing a Fourier Domain Mode Locked (FDML) laser [7].

The optical setup for extended focus OCT imaging is the same as the one previously published [8]. Microcirculation imaging was performed over a 2 x 2mm FOV by acquiring $N = 10$ tomograms at 100 different vertical positions.

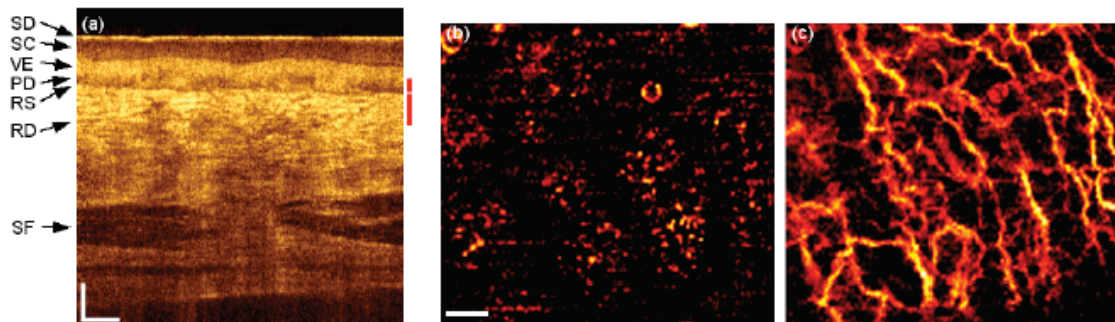


Fig. 2. Healthy skin of the palm. a: OCT tomogram. Red bars indicate depth range for (b) and (c) respectively. SD: stratum disjunctum, SC: stratum corneum, VE: viable epidermis, PD: Papillary dermis, RS: Rete subpapillare, RD: reticular dermis, SF: subcutaneous fat. b and c: 2x2 mm en-face mean projection over depth range indicated in (a). Scale bars indicate 250um in every picture

The result for healthy human skin is shown in Fig. 2 above. Figures 2(b) and 2(c) show the en-face microvascularisation for two different depth ranges, 300 to 350um and 350 to 450um, respectively. A mean projection is calculated over successive axial ranges in order to resolve two different kinds of vasculature: capillary loops feeding the living part of the epidermis, and a deeper planar vascular network that supports the capillary vessels. We further measured the vascular structure for different

pathologic skin conditions: an allergy-induced eczema, seborrhoeic dermatitis, and basal cell carcinoma as a case of non-melanoma skin cancer. The results can be nicely summarized in Table 1 below:

Condition	Effect on microcirculation
Healthy	Organized flat vessels beds with smaller capillary vessels in the upper layers and increased vessel size in deeper skin tissue.
Inflammation	Enlarged blood vessels, in particular capillaries, that indicate increased perfusion.
Basal cell carcinoma	Denser network of unorganized vessels with chaotic branching; larger vessels even close to the skin surface; capillary structure less pronounced and visible.

In conclusion, perfusion and tissue vasculature is an important biomarker for tissue health that can be efficiently assessed by Doppler OCT. Future directions in ophthalmology will certainly include OCT intensity information in order to complement the dynamic flow contrasting also with static information. Tracking systems will further improve the quality of flow contrast images by reducing motion artifacts. Also in case of dermatology the access to functional perfusion data will enhance the diagnostic capabilities of current OCT scanners. Further applications of DOCT can be envisioned in the field of endoscopy. Finally, new light source and detector technology might open the door for novel DOCT methods that allow for even faster vascular imaging over larger field of views.

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DYNAMIC IMAGING OF PROTEIN-PROTEIN INTERACTION IN LIVING CELLS USING EXOGENOUS AND ENDOGENOUS FRET PAIRS*

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**This work is dedicated
to late Prof. Robert M. Clegg (1945–2012),
one of the pioneers in FLIM-FRET Microscopy.*

Abstract. Förster resonance energy transfer (FRET) is an effective and high resolution method to investigate protein–protein interaction in live or fixed specimens. Exogenous fluorophore such as visible fluorescent protein has been widely used as a FRET pair. The contamination involved in FRET microscopy imaging techniques can be removed by using appropriate algorithm. On the other hand, fluorescence lifetime imaging microscopy (FLIM) technique is used to image the protein molecule to avoid any contamination correction. We also discuss the usage of endogenous fluorophore such as tryptophan and NADH as a FRET pair to investigate the metabolic activity.

Introduction

FRET technique is increasingly employed to access the molecular mechanisms governing diverse cellular processes such as vesicular transport, signal transduction and the regulation of gene expression [1]. The protein molecules should be close together within 10nm, the dipole moment of the fluorophore targeted to the proteins should have an appropriate orientation, and the spectral overlap of the donor emission to the acceptor absorption should be >30% for FRET to occur [2-3]. FRET can be used to estimate the distance between interacting protein molecules *in vivo* or *in vitro* using light microscopy systems. Light microscopy techniques including wide-field, confocal and multiphoton microscopy system provides spatial information on the interacting proteins with nanometer resolution [1]. On the other hand, the microscopy technique provides contamination in the FRET signal due to spectral overlap of donor emission to the acceptor absorption. These contaminations have to be removed to make a reasonable interpretation of the FRET data. A number of algorithms have been developed to remove the contaminations in the FRET signal [1, 4-6]. On the other hand, the fluorescence lifetime imaging microscopy (FLIM) approach provides quantitative information with spatial and temporal information of protein-protein interactions [7-9]. In FLIM-FRET technique one follows the change in lifetime value of the donor without and with the acceptor molecules. FLIM methodology has been used in wide-field, confocal and multiphoton based microscopy systems. FLIM is sensitive to the local microenvironment of the molecule but insensitive to the change in its concentration or excitation intensity [7].

FRET Pair

Visible fluorescent proteins (VFPs) have been widely used as a FRET pair in addition to organic dyes. Most widely used FRET pair is Cerulean and Venus. Invention of many color variants generated interest in implementing three protein interactions [10]. Particularly the FRET standards linking the donor and acceptor with amino acids (aa) provided a great help (www.addgene.org) to demonstrate the distance dependence of FRET. Another FRET imaging approach is possible using endogenous fluorophore molecules such as tryptophan and NADH. No fluorophore is required to label these molecules and these molecules provide auto-fluorescence after excitation with appropriate excitation wavelength. Changes in energy metabolism, mitochondrial functions and of reactive oxygen species have been shown to induce alterations in cellular activities which are different in cancer vs. normal cells [11]. Investigation of the metabolic activity at the molecular level would provide detection of cancer at the early stage. The binding of NADH and TRP results in changes in the TRP fluorescence lifetime. This relative quenching of TRP by NADH due to FRET measured by 3-photon FLIM correlates to the level of the cellular metabolic state [12].

FRET Imaging Systems

FRET microscopy system provides spatial and temporal information on the interacting protein in live or fixed specimens. Any light microscopy system can be used for FRET imaging and that includes wide-field, confocal, and multiphoton imaging systems. The FRET pair (donor and acceptor) has to be selected based on the availability of the excitation wavelengths [1]. The energy from donor to acceptor is transferred if the conditions are satisfied. By exciting the donor molecule the FRET signal is collected in the acceptor channel. The acceptor molecule is not excited to collect the FRET signal. For quantitative analysis spectral bleedthrough or contamination correction is necessary. On the other hand, the lifetime imaging system does not require any bleedthrough correction and one can measure the change in lifetime of the donor in the absence and in the presence of acceptor. Time correlated single photon counting (TCSPC) is widely used in FRET imaging. TCSPC required a pulsed laser and a high sensitivity detector. Frequency domain method also used in FRET imaging.

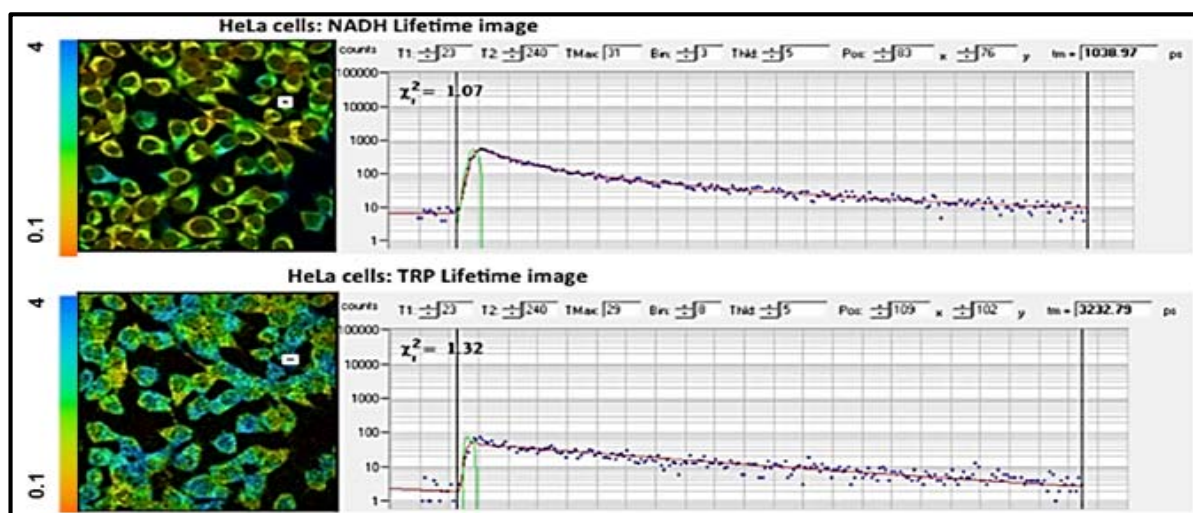


Fig. 1. Two-photon (Ex 740 nm, Em 480/40 nm) lifetime image of NADH and Three-photon (Ex 740 nm, Em 360/40 nm) lifetime image of tryptophan (TRP) were collected using live HeLa cells. Biorad Radiance 2100 multiphoton imaging system; Coherent Ti:sapphire Mira 900 laser; Becker & Hickl SPC 150 and PMT-PMC100-0; Objective lens Nikon 60X W 1.2 NA

In this presentation we describe the usage of FRET methodology using exogenous and endogenous fluorophore as a FRET pair to investigate the C/EBP α dimerization in the living GHFT1 live cell nucleus (2- and 3-color FRET) and the interaction between TRP-NADH in the tumorigenic and notumorigenic human living cells, respectively.

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HIGH PERFORMANCE MOLECULAR IMAGING WITH REAL-TIME MULTISPECTRAL OPTOACOUSTIC TOMOGRAPHY

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Abstract. From a promising new technology optoacoustic imaging is increasingly translating into practical use with numerous biomedical applications continuously emerging. The talk reviews the latest technological developments that put forth optoacoustics as a method of choice for functional and molecular imaging, drug discovery and selected clinical niches.

Despite the ancient discovery of the basic physical phenomenon, it was not until very recently that optoacoustics was first applied to biomedical imaging, experiencing an exponential growth of technical developments and related applications in the last decade [1]. Being a time-resolved signal, a single optoacoustic waveform may provide one-dimensional depth profiling information, similarly to A-mode visualizations in ultrasound imaging. Thereby, the simplest way of acquiring two- or three-dimensional optoacoustic images consists in using linear raster scanning of a spherically-focused ultrasonic transducer along the surface of the imaged object and stacking up sets of the measured signals, a method utilized by most existing optoacoustic microscopy setups [2]. However, scanning imaging systems are generally afflicted with long acquisition times while focused detection inevitably results in out-of-focus artifacts, leading to lack of quantification and overall reduced image quality. Alternatively, optoacoustic tomographic systems have been suggested, based on simultaneous acquisition of optoacoustically-induced signals at multiple locations surrounding the imaged object. For instance, parallel detection of optoacoustic signals with an array of cylindrically-focused ultrasonic transducers has been recently used to render high fidelity cross-sectional images at video rate [3]. The real-time performance has dramatically reduced image artifacts associated with motion, such as heart beat and breathing and enable visualization of various dynamic phenomena *in-vivo*.

In vascularized tissues, highly absorbing hemoglobin manifests good optoacoustic contrast. It is therefore natural for optoacoustics to attain high fidelity images of vascular anatomy, dynamic microcirculation, tumor neovascularization, as well as blood oxygenation levels deep within highly diffuse tissues without introduction of contrast agents [2]. However, imaging of extrinsic photoabsorbing agents may offer important contrast advantage and imaging specificity but would typically require differentiation of these agents on top of spectrally varying background tissue absorption. In response, the so-called multi-spectral optoacoustic tomography (MSOT) technique relies on the spectral identification of chromophoric molecules and particles distributed in tissue over background tissue absorption [4]. Pulses of different wavelengths are used, in a time-shared fashion, whereas the wavelengths are selected to sample a certain spectral characteristic in the absorption spectrum of intrinsic bio-markers and reporter agents of interest. Essentially, when operated at multiple wavelengths, optoacoustics is capable of resolving spectral signatures of chromophores, i.e. it can sense color, or more precisely, listen to color. Due to the powerful combination of multispectral imaging with real-time visualization capacity, several important applications in small animal research, such as visualization of hemodynamic processes, *in-vivo* imaging of kinetics and real-time biodistribution of functional and molecular contrast agents, have recently emerged [5].

At present, the great potential of optoacoustic imaging that was showcased in preclinical research, has encouraged translation of this technology into clinical practice with multiple applications envisioned, from cardiovascular and cancer diagnostics to ophthalmology and endoscopic imaging. One key advantage of the optoacoustic method is its intrinsic ability to deliver complete volumetric tomographic datasets from the imaged object with a single interrogating laser pulse, possibility that does not exist in other imaging modalities. This capacity comes with important advantages, such as ability to dynamically visualize biodistribution, reduce out-of-plane and motion artifacts, and accelerate clinical observations. However, multiple technical limitations related to lack of appropriate ultrasound detection technology, digital sampling and processing capacities hindered so far implementation of volumetric optoacoustic imaging system suitable for dynamic visualization of human pathology in the clinical setting. Furthermore, while small animals are usually fully accessible

for illumination and detection from multiple directions, due to the large dimensions, most parts of a human body can only be accessed from a single side. This imposes limitations on an optimal concurrent arrangement of the illumination and detection components, which are crucial for acquiring the desired quantification performance and image quality for a given application. For instance, adaptation of the common ultrasound linear array probes for optoacoustic imaging remains challenging, mainly due to fundamental differences in tomographic imaging requirements between ultrasound and optoacoustics, resulting in poor imaging performance and lack of sensitivity and quantification [6]. In addition, living objects present a complex target for optoacoustic imaging due to the presence of a highly heterogeneous tissue background in the form of strong spatial variations of scattering and absorption. Extracting quantified information on the actual distribution of tissue chromophores and other biomarkers constitutes therefore a challenging problem. Image quantification is further compromised by some frequently-used approximated inversion formulae.

The talk concentrates on our recent efforts in addressing multiple technical and application-related challenges with the overall goal of translating the MSOT imaging technology into a high performance functional and molecular imaging modality routinely used in both pre-clinical research and clinical practice. All in all, with the compelling advantages of optical imaging, such as highly diverse contrast mechanisms and easy and safe usability, and with imaging performance characteristics that rivals MRI in resolution and nuclear imaging in specificity, it is anticipated for MSOT to play a major role in biomedical research and drug discovery applications. Potentially, it can also play an increasingly important role through clinical trials by offering a method that can yield quantitative markers of treatment and diagnosis while it is not limited by application repetition due to cost or the use of ionizing radiation, within the application areas of the technique, as defined by its penetration ability. Therefore it is expected that MSOT will define several new application areas and will become a method of choice in small animal research and select clinical imaging applications.

Acknowledgements

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HIGH THROUGHPUT, HIGH CONTENT TISSUE IMAGE INFORMATICS

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Abstract. The field of multiphoton microscopy has enjoyed vibrant development over the past two decades. The study of neurobiology is an area where this imaging technology has made major impact. In this presentation, we will provide an overview of the contributions of multiphoton microscopy in neurobiology. More specifically, we will present some recent works on understanding neuron structural plasticity on the dendritic and synapse levels, connectomics of neuronal network, and molecular regulation of peripheral nerve regeneration. The limitation of our current approach in studying neuronal structures has further motivated us to image with higher resolution by employing structured light based wide-field two-photon excitation, to enable higher content imaging by implementing new spectral and lifetime resolved modalities, and to image faster by developing simultaneous parallelized 3D imaging.

In this presentation, we will describe three major classes of new image cytometry systems for imaging (1) cells in 2D cultures and thin matrix, (2) 3D *in vivo* tissue systems, and (3) 3D whole organs *ex vivo*. All three systems have their respective strengths and weaknesses and are designed to optimize imaging of specific specimens, mostly limited by their thickness. Specific examples in neurobiological systems will be described.

1. Structured light 3D imaging cytometry for cell cultures

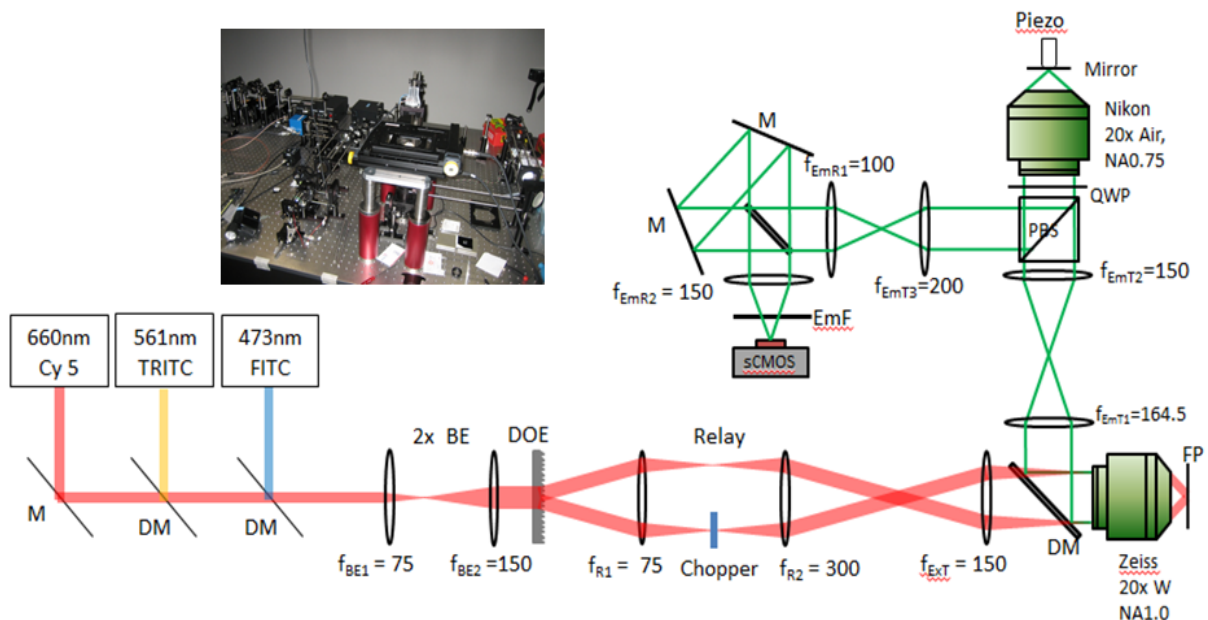


Fig. 1. Structured light 3D image cytometer

2. Temporal focusing multiphoton image cytometer for *in vivo* tissues

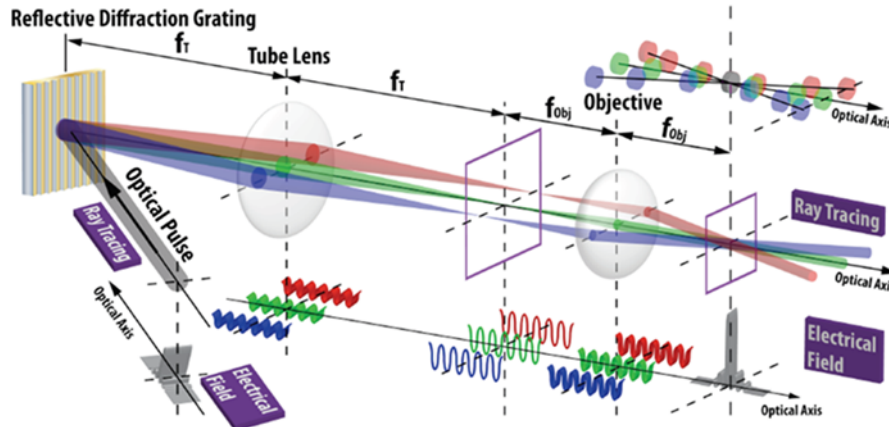


Fig. 2. Design of temporal focusing multiphoton wide-field image cytometer

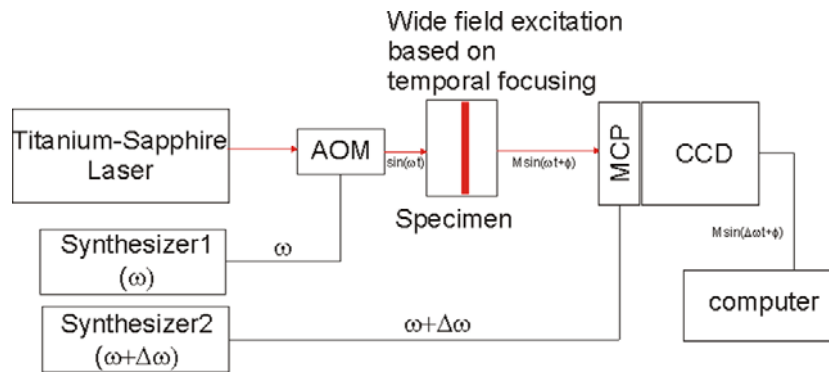


Fig. 3. Adding fluorescence lifetime resolved wide field imaging

3. Multifocal multiphoton image cytometer for *ex vivo* whole organ imaging

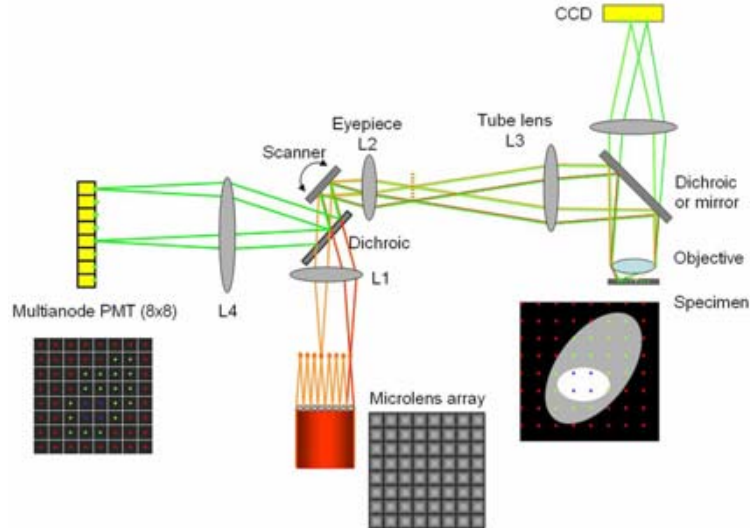


Fig. 4. Multifocal Multiphoton image cytometer

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ARTIFICIAL CHROMOSOMES FOR REGENERATIVE MEDICINE AND GENE THERAPY

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Abstract. Studying molecular mechanism controlling pluripotent stem cell self-renewal and differentiation is a highly relevant pursuit, however, there is a clear need to develop novel approaches that would ensure rapid and safe introduction of these unique cells to clinics, beneficial for human health. The most recent and exciting topic of our research aimed at the clinical application is dedicated to a tumor-free use of embryonic stem (ES) and induced pluripotent stem (iPS) cells in tissue-replacement therapies. The major goal of current grant proposal is to bolster genetic sensitization method previously developed by us by putting it on the non-integrative platform. This modification should basically eliminate the risks of both insertional mutagenesis by suicidal DNA-cassette, as well as epigenetic silencing of this cassette following its genome integration. We intend to achieve this goal by deploying the human artificial chromosomes (HACs), and further extend the approach, which will combine ES/iPS-based tissue-replacement, genetic sensitization, and gene therapy technologies, onto treatment of recessive hereditary diseases.

Addressing the safety issues of ES/iPS-based regenerative medicine

Pluripotent stem cells, such as blastocyst-derived ES cells and differentiated cell-derived iPS cells, hold great promise in human tissue-replacement therapies because they possess the capacity for unlimited self-renewal and the ability to differentiate *in vitro* into virtually any cell type of adult organism. However it is also known that ES/iPS cells can form teratomas upon injection into immune-matching or immune-compromised animals. Even though teratomas are considered to be non-malignant tumors, their rapid uncontrolled growth in space-constrained vital organs such as brain or spinal cord would normally be lethal. On the other hand, following differentiation of *in vitro* cultures in the direction of particular tissues, residual undifferentiated ES/iPS cells always persist to some degree. So there is an absolute requirement in developing technologies for the highly reliable methods of removal of these cells following cell differentiation. Several approaches, such as cell sorting or positive selection (via lineage-specific genetic markers or drug selection) have been proposed to address this critical safety issue although with serious limitation. Clearly, there is a need to develop novel approaches to address the issue of ES/iPS cell tumorigenicity.

Recently we have proposed an approach for genetic sensitization that allows addressing this critical issue [1]. This approach relies on negative selection of ES/iPS cells, using a suicide gene, thymidine kinase (TK) gene, under control of a short ES/iPS-specific Oct4 enhancer (2A2B) and tk minimal promoter/moiety. To our knowledge, the 2A2Btk moiety represents the shortest known regulatory element sensitive to the differentiation status of ES and iPS cells. In addition, the relatively small size of this cassette allows stable genome integration via lentiviral vectors. The developed approach allows robust elimination of residual ES/iPS cells by gancyclovir (GCV) treatment not only in mixed populations of cultured differentiated cells *in vitro*, but also *in vivo*, following direct injection of undifferentiated ES/iPS cells into immune-compromised or immune-compatible recipient mice (manuscript in preparation). We have illustrated this drastic way of depleting ES cells (previously unimaginable in the absence of the firm control over teratomas) by showing both lymphoid and myeloid cell type reconstitution of peripheral blood in lethally irradiated after transplantation of undifferentiated ES cells and subsequent GCV treatment that suppressed teratoma formation. Besides being useful for teratoma growth control, the developed approach might also find important clinical application in tissue-replacement therapies with cell types that cannot readily be derived from ES/iPS cells in sufficient amounts *in vitro*, for example, hematopoietic cell types (see above) or insulin-producing beta-cell of pancreas.

There are two major drawbacks in the proposed sensitization method, which need to be addressed before introducing this method into clinics. The first problem is a possibility of spontaneous epigenetic silencing of the suicidal cassette integrated into host genome (both as a transgene or provirus), resulting in a weakened control over teratomas. Although HIV-derived lentiviruses are considered to efficiently resist epigenetic silencing in the majority of cell types, this is clearly not the case with ES/iPS cells. For example, a high rate of lentivirus silencing in newly derived rat iPS cells was observed. This problem could be partly addressed by applying continuous positive selection pressure on the sensitized ES/iPS cells with puromycin. However, in certain scenarios, such as described above, transplantation of undifferentiated

ES/iPS cells, this selection pressure is to be relieved for several days (which might be sufficient for cassette silencing), allowing these cells to perform a homing to a damaged organ and differentiate into a desired lineage. The second problem of the suicidal approach is associated with insertional mutagenesis caused by random integration of suicidal transgene or lentivirus into ES/iPS cell genome, resulted in an elevated risk of neoplasia. This scenario is very common, for example, when bone marrow cells are transplanted, following genome integration of therapeutic genes.

We aim to demonstrate that HACs are an ideal vector system that is devoid of the problems associated with insertional mutagenesis and epigenetic silencing because they do not integrate into host genome and are extremely stable. Alphoid TetO-HACs are therefore used to deliver the suicidal cassette and its more advanced derivatives into iPS and/or ES cells to allow tackling hereditary diseases via teratoma-free tissue replacement.

Developing novel approaches to iPS cell- and HAC-based gene therapies of human hereditary diseases

HAC is a formidable vector system that has an enormous capacity, does not modify host genome, and is extremely stable inside host cells [2]. As such the system is highly suitable deliver and stably maintain the expression of therapeutic genes. On the other hand, due to a relatively large size of HAC they have some disadvantages. First, contrary to viruses or plasmids, they cannot be readily amplified. Second, contrary to viruses, HACs cannot be readily delivered to target tissues or organs. On the other hand, the only currently existing method for HAC delivery has been carried out solely on cultured cells via very inefficient and demanding method of MMCT. Therefore HAC delivery to target organs or tissues can be performed via HAC-carrying cells that will subsequently incorporate or replace these organs or tissues. Adult stem cells are possible candidates for such carriers. For example, hematopoietic stem cells would be the choice to deliver therapeutic HACs lymphoid and myeloid cell types. The HAC-carrier cells have to meet three criteria: they must be clonogenic, i.e. capable to expand from a single cell (MMCT procedure requires a clonal selection); they must retain a significant self-renewal potential to allow production of a clinically relevant cell mass, and third, they have to demonstrate genetic and epigenetic stability. For very few exceptions, adult stem cells or immortalized somatic cells do not meet these criteria. All three criteria, however, are readily met by pluripotent stem cells, such as iPS or ES cells, which have the capacity for virtually unlimited self-renewal with the retention of their differentiation potential in vitro. These unique properties make iPS and ES cells ideal HAC-carriers. On the other hand, HACs bring into the stem cell field their unique features, such as enormous capacity, autonomy, high stability and, as featured by alphoid-TetO HACs, the possibility to be extruded from the cells in an inducible manner.

Thus iPS/ES cell and HAC technologies remarkably compliment each other, resulting in a spectrum of possible output applications that might address several important issues of regenerative medicine and gene therapies at a completely new level. Two of these issues, related to the safety of iPS cell derivation and iPS/ES cells transplantation, have been highlighted. Yet another important issue regards gene therapy of recessive hereditary diseases of human. In our research we deploy the gene therapy of hereditary diseases on mutant mice, modeling some socially significant or rare genetic diseases. We will create a series of gene therapy HACs (gtHACs), expressing wild-type alleles of disease-related genes, both as mini-genes, i.e. open reading frames (when possible, codon-optimized ones) driven by ubiquitous or tissue-specific promoters or as the whole genomic locus including intronic, intergenic, and subtle regulatory elements.

We expect that establishing HAC- and iPS cell-based gene therapy protocol(s) will open broad possibilities for the treatment of the whole spectrum of hereditary recessive diseases in human via the therapeutic cycle which might ultimately incorporate HAC-mediated iPS cell derivation and genetic sensitization. We thus expect a profound effect of this work in both regenerative medicine and gene therapies.

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FUNDAMENTALS AND ADVANCES OF TISSUE OPTICAL CLEARING

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Abstract. The state-of-the-art of research in the field of tissue optical clearing in application to enhanced optical imaging and monitoring of drug delivery in the framework of receiving of more precise and valuable information from reflectance spectroscopy, polarization measurements, Raman spectroscopy, confocal microscopy, and optical coherence tomography (OCT), as well as from nonlinear spectroscopies, such as two-photon fluorescence, second harmonic generation (SHG) and terahertz spectroscopy are presented. *In vitro*, *ex vivo*, and *in vivo* studies of human tissues and blood will be presented. Optical clearing agents and delivery techniques, as well as tissue enhanced permeation, will be discussed. Some important applications of optical immersion technique in dermatology, ophthalmology, gastroenterology, and some other medical fields will be demonstrated.

The main limitation of optical imaging and spectroscopic techniques is caused by a strong light scattering, which significantly lowers the image quality and probing depth. Diffuse optical tomography and biopsy, optical coherence tomography (OCT), confocal microscopy, SHG imaging, multi-photon spectroscopy, polarization imaging, Raman spectroscopy, Doppler and speckle blood flow imaging techniques are very powerful modalities in investigation of tissues and disease diagnostics. However all these techniques are suffer strongly from light scattering. The tissue immersion optical clearing (IOC) technique based on application of biocompatible optical clearing agents (OCAs) is an attractive method for controlling of optical properties of a variety of tissues due to strong but reversible reduction of tissue scattering ability. It provides many benefits for successful application of optical methods in biomedicine [1-6].

The IOC represents a promising approach to increase the imaging depth of nonlinear spectroscopies, confocal microscopy, and OCT for which this is a critical parameter. The improvement of light penetration depth and image contrast in tissues and blood achieved at application of such OCAs, as glycerol, glucose, ethylene glycol, polyethylene glycol, propylene glycol, polypropylene glycol, will be demonstrated for *in vitro* and *in vivo* studies.

Mechanisms behind IOC are refractive index matching of tissue structures and interstitial fluid, as well as better ordering of tissue structural components due to: 1) agent diffusion into the tissue (mostly into interstitial space), 2) tissue dehydration, and 3) tissue shrinkage.

For optical diffusion imaging, in-depth blood flow monitoring using diffusion wave spectroscopy, and other principally scattering-based techniques, reduction of scattering of the upper layers of tissues also could be useful to provide more flexibility in getting quantitative information about tissue lesions and functioning.

A new approach for IOC based on production of self-clearing (metabolic products) agents inside tissue and blood will be discussed. Two examples of self-clearing of the adipose tissue under its photodynamic/photothermal treatment and adipocyte lipolysis [7], as well as local lysis of red blood cells (RBCs) in blood stream [8], will be analyzed. Optical clearing experiments with the products of cell lipolysis (free fat acids (FFAs), glycerol and water) and RBC lysis (hemoglobin) will be analyzed. For example, due to light-induced fat cell membrane porosity, the intercellular content of the cell (FFAs) percolates through the arising temporal pores into the interstitial space and due to the refractive index matching effect the optical medium becomes optically more homogeneous and more transparent to light.

The diffusivity of glucose-water solutions at different concentrations in muscle tissue and dependence of solution diffusivity on soft and hard tissue hydration [9] and glycation will be analyzed from the point of view of cancerous and diabetes impact on tissue diffusivity as a novel marker for these diseases detection and monitoring.

OCA delivery techniques, as well as tissue enhanced permeation, will be discussed. Some important applications of IOC technique in dermatology, ophthalmology, gastroenterology, and some

other medical fields will be demonstrated. The specificity of IOC in terahertz range will be also discussed.

A mechanical stress on a soft tissue in the form of compression or stretching causes a significant increase in its optical transmission due to less overall light scattering and blood absorption [10-14]. Askar'yan [10] was the first to study the propagation of a laser beam through the soft tissue phantoms and human palm at mechanical compression. The reduction of extinction coefficient after tissue compression and prolongation of optical clearing effect after removing pressure for some time interval were successfully demonstrated. For living tissues the major mechanisms behind this phenomenon are: 1) increased optical tissue homogeneity due to removal of blood and extracellular fluid from the compressed site; 2) more close packing of tissue components leading to constructive interference (cooperative) effects; and 3) less tissue thickness.

The estimation of biomechanical properties of tissue is critical to many areas of the health sciences, including monitoring of the tension in wound closures, skin flaps, and tissue expanders. Optical and especially OCT techniques are very helpful in such studies.

As a well-blood-supplied tissue, skin spectral properties can be effectively controlled by applying an external localized pressure in *in vivo* experiments when UV induced erythema (skin redness) is developed [14]. For that particular case, extra blood in the skin dermis coming due to physiological reaction on UV light is pushed out by compression, thus providing better light transmittance within the bands of hemoglobin even the induced erythema without compression is rather strong.

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Optical Bioimaging

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THE INFLUENCE OF COMPRESSION AND TEMPERATURE REGIME ON FORMATION OF HUMAN SKIN OCT-IMAGES

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Abstract. Optical coherence tomography is a modern method for visualization of internal structure of biotissue up to several millimeters in depth with resolution down to several micrometers. However, external conditions, such as compression or abnormal temperature regime, can distort the diagnostic picture in an OCT-image. We show that application of compression to human skin induces a decrease in the contrast of stratum corneum-epidermis junction and an increase in the contrast of epidermis-dermis junction; preliminary change of biotissue temperature induces additional changes in the contrast of these junctions with opposite effect in cases of heating and cooling.

Optical coherence tomography (OCT) is a modern method of visualization of internal structure of biotissue at the depth up to several millimeters with resolution down to several micrometers [1-3]. This method is based on low coherence interferometry and uses information from photons backscattered from inhomogeneities of refractive index within biotissue. Therefore intensity of OCT-signal depends on distribution of optical properties in biotissue. By changing the distribution of optical properties one induces changes in backscattering from biotissue and, respectively, in OCT-signal. Different external conditions, such as compression or abnormal temperature regime, may cause changes in refractive index distribution. Thus, an OCT-image is sensitive to these effects and study of the influence of external conditions is important for accurate interpretation of OCT-images and proper diagnosing.

In this work we compare OCT-image formation in three different cases: under low compression, under high compression and under high compression with preliminary change of biotissue temperature.

The study was performed *in vivo* on human volunteers of different age (20-50 years old). For pressure control we have developed a special dynamometer which can be conjugated with the OCT probe of the device. When evaluating the pressure of the probe surface to the tissue we supposed that it is distributed uniformly over the whole probe surface, and its value was obtained as a ratio of the force measured by the dynamometer to the surface area. OCT images of skin were obtained each 5 seconds after compression start for 7 minutes. The pressure to skin was kept constant during the whole time of observation and equal to 0.04 N/mm² in the low pressure case (providing full contiguity of probe surface with studied biotissue) and 0.21 N/mm² in high pressure case (maximal pressure which does not induce unpleasant feelings in patient). The pressure was lower compared to that used in our previous study [4] to increase comfort for volunteers. At the next stage we performed preliminary heating or cooling of skin before application of pressure.

Figure 1 demonstrates the increase of the time dependence of the contrast of epidermis-dermis junction (Fig. 1a) and stratum corneum-epidermis junction (Fig. 1b) in the case of compression without preliminary temperature variation, after preliminary cooling and preliminary heating of skin. In all cases the time dependence demonstrates the same dynamics for both junctions due to compression of skin that leads to sticking of horny dry cells and redistribution of extracellular liquid. The former factor leads to the decrease in scattering from stratum corneum and to the decrease of the contrast of stratum corneum-epidermis junction; the latter factor leads to increased scattering from dermis layer and increased contrast of the epidermis-dermis junction, respectively. Long application of compression causes appearance of dark areas in the obtained OCT images, that are most likely associated with an interstitial or intracellular water inflow to the observed region. Changes in the epidermis are not observed. For more detailed interpretation of the mechanisms of the compression effect on OCT-images of human skin a set of Monte Carlo simulations were performed. Results of simulations are in agreement with experimental results.

After preliminary cooling the spasm of blood vessels in dermis occurs. This factor causes a decrease of relative blood content and a decrease in absorption in the region under action. The decrease in absorption in turn causes increased OCT-signal from dermis layer and, consequently, increased contrast of the epidermis-dermis junction. As a result, during the whole observation time the contrast in the case of preliminary cooling (Fig. 1, ♦) is higher compared to that without cooling (Fig. 1, ▲).

The preliminary heating induces two mechanisms affecting both the considered dependences. The former is vasodilatation causing an increase in relative blood content and the corresponding increase of local absorption that leads to decreased epidermis-dermis junction contrast. The latter is sweating of skin in the area of action that leads to filling spaces between horny dry cells with sweat and the corresponding decrease in scattering from stratum corneum. This mechanism causes a decreased contrast of stratum corneum-epidermis junction. Accordingly, during the whole observation time the contrast in the case of preliminary heating (Fig. 1, ■) is lower compared to that without heating (Fig. 1, ▲).

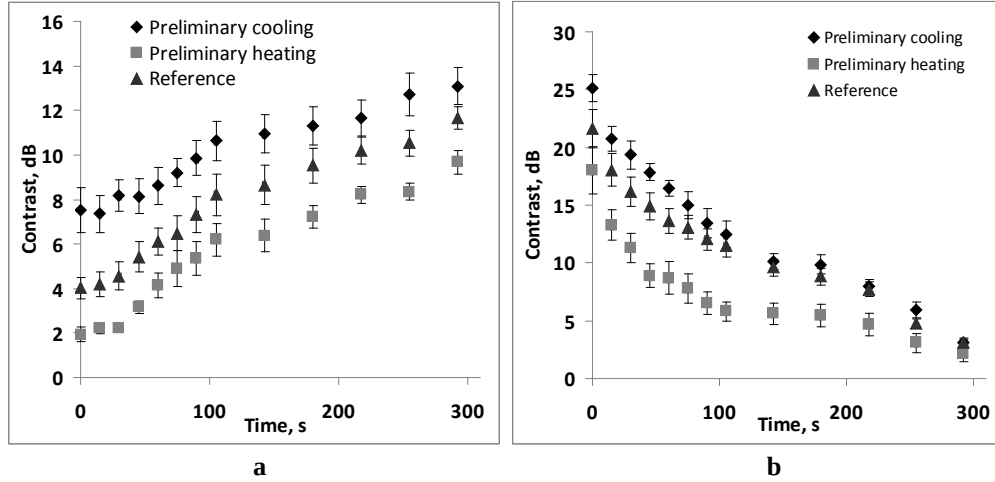


Fig. 1. Time dependence of contrast of epidermis-dermis junction (a) and stratum corneum-epidermis junction (b) without temperature effect, preliminary cooling and preliminary heating

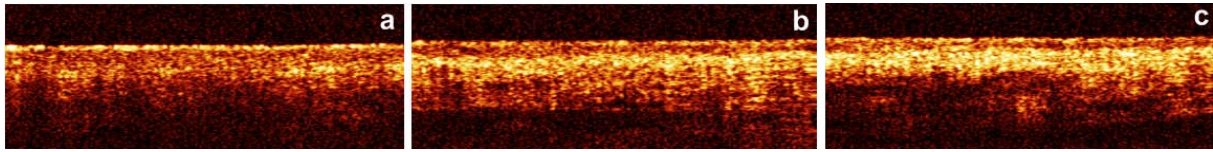


Fig. 2. Typical OCT-images of human thin skin: without temperature action immediately after compression start (a), 7 minutes after (b) and 7 minutes after with preliminary cooling (c)

Thus, we show that biotissue compression and changes in biotissue temperature regime lead to changes in interlayer junctions contrast. Application of compression to human skin induces a decrease in the contrast of stratum corneum-epidermis junction and an increase in the contrast of epidermis-dermis junction. Preliminary change of biotissue temperature induces additional changes in the contrast of these junctions with opposite effect in cases of heating and cooling. This behavior can be explained by different mechanisms of tissue response.

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CAPABILITIES OF OPERETTA SYSTEM FOR HIGH-CONTENT SCREENING

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Abstract. Acquiring of data and image analysis of the samples can be a time consuming and challenging process. The use of the Operetta system for High Content Screening provides analysis of a large number of samples by automated imaging. The use of special marker proteins with the Operetta system provides selection of different types of cell subpopulations, cell cycle progression, cytoskeleton rearrangements, receptor internalization, co-localization and protein accumulation in spots, and many others. We applied the Operetta system for screening library of small molecules that potentially can inhibit E3 ubiquitin-protein ligase Mdm2.

A growing number of cancerous disease cases evoke the necessity for search of new approaches for cancer treatment. One of the perspective approaches is a targeted therapy. This approach implies the apoptosis of cancer cells with minimal side effects on normal somatic and germ cells. Most of mutations occur in genes that are involved in signal transduction pathways, cell cycle progression, DNA replication, DNA repair and apoptosis. Apoptosis of defective cells is an important step for prevention of cancer development therefore it attracts a particular interest. One of the key genes which promote apoptosis induction is transcription factors that belong to p53 protein family (p53, p63, p73). A half of patients with cancerous diseases carry mutation in *TP53* gene.

It is known that cellular functions of p53 can be controlled by ubiquitin-dependent pathway of degradation. The covalent modification of p53 by ubiquitin is performed by specific E3-ubiquitin ligases Mdm2 and Itch. After ubiquitination, p53 is recognized by the 26S proteasome and proceeds to degradation. We suppose that inhibition of Mdm2 and Itch will predispose sensitivity of cancer cells to chemotherapeutic drugs and apoptosis as a result of p53 stabilization. Small molecules for inhibition of Mdm2 and Itch functions can be used for p53 stabilization and suppression of cancer cells growth. The main approach that we apply is the use of fluorescently targeted proteins assay that allows direct detection of proteins of interests. The use of the Operetta system for screening library of small molecules allows us to find compounds that can inhibit E3 ubiquitin-protein ligase Mdm2.

The Operetta system is a multifunctional device. The system allows getting more that 100 000 image sets per day with different resolution, and it is able to analyse both fixed and live cells. A large set of fluorescent filters provide an opportunity for simultaneous analysis of at least four different fluorescently targeted proteins. In our case, we analysed fluorescence of fluorescently targeted protein p53 and fluorescent reporter plasmid that allows direct detection of the effect of small molecules on proteins of interests.

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GREEN, COMPACT DIODE LASER-BASED SYSTEMS FOR BIOPHOTONICS APPLICATION

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Abstract. Diode lasers are by far the most efficient lasers currently available. With the ever-continuing improvement in diode laser technology, this type of laser has become increasingly attractive for a wide range of biomedical applications. Compared to the characteristics of competing laser systems, diode lasers simultaneously offer tunability, high-power emission and compact size at fairly low cost. Therefore, diode-based lasers are increasingly preferred in important applications, such as photocoagulation, optical coherence tomography, diffuse optical imaging, fluorescence lifetime imaging, and terahertz imaging. In this talk, focus will be on the green laser system and, as an example, its applicability in optical coherence tomography.

Summary

A commonly used light source within biophotonics diagnostics and imaging applications is mode-locked Ti:sapphire lasers offering ultrashort pulses with correspondingly large bandwidth. Ti:sapphire lasers have their main absorption band in the blue-green spectral range. Currently, the main pump laser for Ti:sapphire lasers is frequency doubled diode-pumped solid state (DPSS) lasers. Such lasers typically provide high output power with excellent beam properties at a wavelength of 532 nm. Alternatively, a more cost effective and compact pump laser would be to use the direct emission of diode lasers. However, direct diodes are currently limited in terms of output power.

A frequency doubled diode laser constitutes a possibly better suited pump laser in the 1 to 10 W range for Ti:sapphire lasers [1]. Frequency converted diode laser systems remove the need for a high finesse laser resonator, thus enabling compact laser systems with low requirements on positioning of components. One further advantage of diode lasers is that the typical relaxation oscillation frequency of diode lasers is in the GHz range and noise originating from relaxation oscillations will be cancelled out by the excited state lifetime in the Ti:sapphire material.

In its simplest realization, the doubled laser is schematically shown in Fig. 1 [2]. Light from the tapered diode laser is collimated in the fast axis by an aspherical lens with a focal length of 3.1 mm. Due to astigmatism in the laser, the fast axis collimating lens focuses the light in the slow axis and an additional cylindrical lens with 15 mm focal length is used to collimate the beam in the slow axis and correct for astigmatism. The second half-wave plate is used to adjust the polarization for optimum SHG in the PPMgLN crystal. A spherical lens is used to focus the light into the PPMgLN crystal. The footprint is 9 cm by 15 cm. From the above-mentioned experimental setup, it is possible to obtain output power at 532 nm in the range of 1.3 W to more than 2.1 W. Further increase in output power is possible via either spectral beam combining

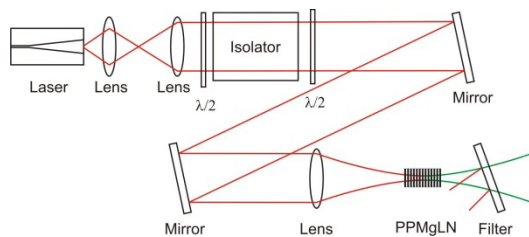


Fig. 1. Schematic of green laser

followed by sum-frequency generation [3] or so-called cascaded single-pass doubling. Recent data suggests a two-fold increase in green output power for the same input power.

As an example, the above-mentioned system (1.4 W at 531.2 nm with beam propagation ratio M^2 of 1.3 and 1.4 along the fast and slow axes, respectively) is used as pump source for a Ti:sapphire laser. The beam is collimated and focused into the Ti:sapphire crystal. The Ti:sapphire crystal, positioned at Brewster angle, is 3 mm long and has a figure of merit >150 and an absorption coefficient of 4.5 cm^{-1} . The laser oscillator is an X-folded cavity with several extra folds to minimize the footprint and several mirrors in the cavity are dispersion compensating. Two curved mirrors with 50 mm radius of curvature surround the Ti:sapphire crystal and the cavity generates an $18 \text{ }\mu\text{m}$ beam waist inside the crystal. A mirror with 3 % transmission is used for output coupling. The total cavity length is about 1.75 m giving a repetition rate of approximately 80 MHz.

Optical coherence tomography (OCT) is a well-established imaging technique enabling depth resolved images with high resolution [4, 5]. The resolution is determined by the bandwidth of the light source, thus, the wide bandwidth of a mode-locked Ti:sapphire laser makes it an attractive source. The main limitation of using Ti:sapphire lasers in OCT systems is the relatively high cost of such lasers, mainly determined by the DPSS pump sources used. A shift of pump source to frequency doubled tapered diode lasers may provide access to lower cost and more compact Ti:sapphire lasers in the future. Below we show initial results of OCT imaging of the retina and skin, respectively, using a Ti:sapphire laser pumped by a frequency doubled tapered diode laser [6]. The OCT system was a modified Spectralis OCT device (Heidelberg-Engineering) with built-in fixation target and eye-tracking device.

The Ti:sapphire laser is adjusted to provide stable mode-locked operation with an output power of 80 mW at 1.4 W pump power with spectral width of 90 nm (FWHM) and >200 nm full width. This provides an axial resolution of less than 4 μm . The light from the Ti:sapphire laser is coupled to an optical fiber and the output power from the fiber is regulated to give a maximum of 0.8 mW at the cornea. A scanning reference arm provides the depth information and a grating based spectrometer is used for monitoring the laser spectrum. Control hardware regulates the fixation light, scanning laser ophthalmoscope and eye-motion correction. Tomograms of the retina in two different subjects are shown in Fig. 2 [6]. Furthermore, tomograms of healthy human skin are shown (same light source, but different beam delivery system).

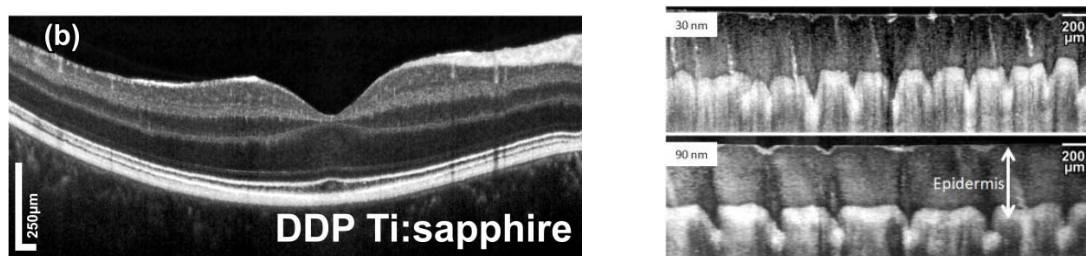


Fig. 2. Left: OCT of healthy retina using the diode-pumped Ti:sapphire. Upper right: OCT of skin using 30 nm bandwidth (FWHM). Lower right: OCT of skin at 90 nm bandwidth (FWHM)

In summary, frequency doubled tapered diode lasers show great potential for many applications within biophotonics. Demonstration of several Watts of output power in the green spectral range from purely diode laser based laser systems facilitates widespread application. Of particular interest is direct pumping of mode-locked Ti:sapphire lasers to generate ultrashort pulses from highly compact and cost-effective laser systems. Biophotonic imaging modalities, such as optical coherence tomography and multi-photon imaging and combinations thereof, may benefit from such laser systems, thus becoming widely applicable.

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MINIATURE MOTORIZED CATHETER FOR OCT, DEPTH RESOLVED FLUORESCENCE AND POLARIZATION SENSITIVITY

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Abstract. We present a miniature motorized endoscopic probe for Optical Coherence Tomography with an outer diameter of 1.65 mm and a rotation speed of 3,000 – 12,500 rpm. The probe has a motorized distal end which provides a significant advantage over proximally driven probes since it does not require a drive shaft to transfer the rotational torque to the distal end of the probe and functions without a fiber rotary junction. In addition, we present a new method for high-resolution, three-dimensional fluorescence imaging. We obtain depth information without the necessity of depth scanning. This method is ideal to be integrated with OCT endoscopy.

1. Introduction

Optical Coherence Tomography (OCT) is a powerful optical technique for high resolution in vivo imaging of tissue morphology [1]. The Optical Frequency Domain Imaging (OFDI) implementation of OCT has shown great potential for comprehensive in vivo imaging of large volumes [2]. A critical research topic in OFDI is the development of high speed high resolution miniature probes for endoscopic in vivo imaging. Optical fiber and gradient-index (GRIN) lens designs are commonly used to deliver and focus light at the distal end of the catheter [3]. Referring to the direction of the imaging beam relative to the catheter itself, fiber based miniature OCT probes can be roughly classified into two categories: forward-viewing catheters, in which the imaging beam exits along the direction of the catheter; and side-viewing catheter, in which the imaging beam exits perpendicularly to the direction of the catheter [4]. While forward-viewing catheters play a role in fields such as bladder and ophthalmic imaging [5-6], side-viewing catheters are utilized widely for imaging within hollow organs, from arteries to esophagus as well as colon and lung [7-10].

For rotational side-viewing OCT catheters, circumferential scanning can be implemented in two ways [4]: proximal scanning or distal scanning. For proximal scanning catheters, a motor placed outside the human body at the proximal end transfers rotational torque to the distal end by a drive shaft. A fiber optic rotary junction (FORJ) couples the light from the stationary to the rotating distal end. This approach enables thin probe designs down to 300 μm diameter [11]. However, a rotating flexible fiber in a stationary sheath commonly creates non-uniform rotation distortion (NURD) originating from friction in (sharp) bends [12]. Moreover, commercially available fiber optic rotary junctions for single mode fibers cannot rotate faster than 5000 rpm. Distal scanning probes use a miniature scanning module, for instance, a micromotor at the distal end of the probe to carry out circular scanning [13]. This design, though challenged by several engineering difficulties, has several advantages. A fiber rotary junction becomes obsolete. Drive shaft friction induced non-uniform rotation distortion can be avoided and higher scanning speeds can be achieved. Furthermore, such designs enable the possibility of combining OCT with other optical imaging techniques in a multimodality system, in which more complicated fiber designs such as double clad fibers may be used without the complication of a FORJ [14].

In this paper we present a miniature probe that integrates a micromotor in the distal end. Together with our OFDI system, we are able to acquire 3D data continuously at a speed of 208 frames per second. Results of ex vivo pig lung imaging experiment are demonstrated. The outer diameter of the probe is 1.65 mm.

Fluorescence microscopy has revolutionized biology and biomedicine during the past decades through the ability to specifically label and image cell structures or proteins of interest. With the development of clinically approved near-infrared fluorescent probes conjugated to therapeutic or diagnostic agents, a similar development is expected in the coming years in clinical medicine [15-19]. To bring these translational advances in fluorescence labeling to clinical practice, however, requires a new class of miniature endoscopic imaging systems that will enable clinicians to rapidly visualize the three-dimensional distribution of fluorescence labels *in situ*.

Self-interference fluorescence microscopy (SIFM) extends the capabilities of confocal microscopy by providing depth sensitivity much better than the axial spot size. Fluorescence self-interference has been explored before [20-24], but these approaches were based on a transmission configuration and

therefore could not easily be incorporated into conventional or miniature imaging systems. Our SIFM method addresses this challenge by collecting the fluorescence in the backward direction and phase-encoding the depth information through the use of a phase plate (e.g., a glass plate with an opening) placed into the beam path. This plate presents the photons emitted by a fluorophore with two alternative paths that then interfere upon coupling into a single mode fiber that acts as a pinhole. The path length difference between the two paths gives rise to depth-dependent interference as a function of wavelength, where moving the fluorescent source axially through the beam waist causes the wavelength-dependent interference pattern to undergo a phase shift. The lateral resolution in SIFM is determined by both the beam waist and the fiber core aperture size, similar to confocal microscopy.

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CLINICAL APPLICATIONS OF NON-INVASIVE OPTICAL MONITORING OF TISSUE METABOLISM

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Abstract. Tissue oxygen delivery is intimately dependent on blood flow, concentration, and oxygen saturation. However, these important parameters are currently difficult or impossible to measure non-invasively in critically ill patients. Diffuse optical techniques provide a window into tissue hemodynamics using tools which can be integrated into intensive care units and applied to fragile patients. Our current studies include serial measurements of hemodynamics in patients following pediatric stroke and corrective surgery for congenital heart defects. Ultimately, we seek to provide tools to permit physicians measure the effects of their interventions on cerebral oxygen perfusion.

Diffuse Optical Spectroscopy (DOS) utilizes light in the near-infrared (NIR) between ~650 and 950 nm [1]. This window of low optical absorption permits measurement of chromophore concentrations and scattering in thick biological tissues (~cm). Local concentrations of water, lipid, oxy-, deoxy-, and total-hemoglobin, as well as blood oxygen saturation, and scattering are readily derived from DOS measurements of muscle, brain, breast, and other tissues. Additionally, recent work at the University of Pennsylvania and elsewhere has expanded the diffuse optical portfolio include Diffuse Correlation Spectroscopy (DCS), permitting measurement of microvascular blood flow with NIR light. These non-invasive low-risk diagnostics are emerging as useful clinical tools, especially in detection & monitoring of cancer and assessment of cerebral function & injury.

Our recent work has applied diffuse optics to answer pressing clinical questions in the critically ill child. A sufficient oxygen supply to the brain is vitally important, as brain ischemia has significant impact on a patient's future quality of life, yet cerebral oxygenation is not routinely monitored in the intensive care unit. Cerebral blood flow is currently measured only occasionally, if at all, in the larger vessels (e.g., with transcranial doppler ultrasound). Current practice utilizes surrogate measurements of cerebral oxygenation (e.g., blood lactate concentration, which provides information on systemic aerobic metabolism). However, these metrics may be misleading in the critically ill with impaired circulation (e.g., due to congenital malformations) or impaired autoregulation (e.g., due to brain injury).

Results from this research hold potential to alter clinical practice by providing continuous feedback to clinicians on the metabolic condition of the brain. This work will provide hitherto unavailable assessment of the efficacy of therapeutic interventions in maintaining cerebral blood flow sufficient to support oxidative metabolism at the bedside. Ultimately, our approach may permit physicians to individualize treatment to maximize benefit and minimize risk to both pediatric and adult patients.

We have applied DCS to measure the efficacy of postural manipulation to enhance blood flow in pediatric ischemic stroke. Durduran and colleagues [2] have previously observed an apparent "paradoxical" response to lowering the head of bed in ~25% of his adult subjects, in which the blood flow in the infarcted hemisphere was reduced when the patient was supine. Similarly, in our first case (Fig. 1), we observed only a transient change in blood flow when the patient was lowered from a reclining to supine position. In this patient, lowering the head of bed appeared to produce only a clinically insignificant transient increase in blood flow. Even the seemingly-benign intervention of lowering a child's head-of-bed angle can raise the risk of fluid aspiration and lead to significant complications; in this particular child's case, a supine position may not be beneficial. We seek to provide real-time cerebral hemodynamic monitoring to permit individualized care and, ultimately, more aggressive clinical interventions (e.g., pharmacological agents to raise blood pressure).

In our experience, diffuse optical devices can easily be integrated into a patient's bedside monitoring equipment without disrupting clinicians or other devices, even in a cardiac or pediatric intensive care unit. In addition to the stroke study discussed above, we have utilized these technologies to monitor neonates following surgical correction of congenital heart defects, catheter-based procedures, and pilot studies of sleep apnea in children with Down's syndrome.

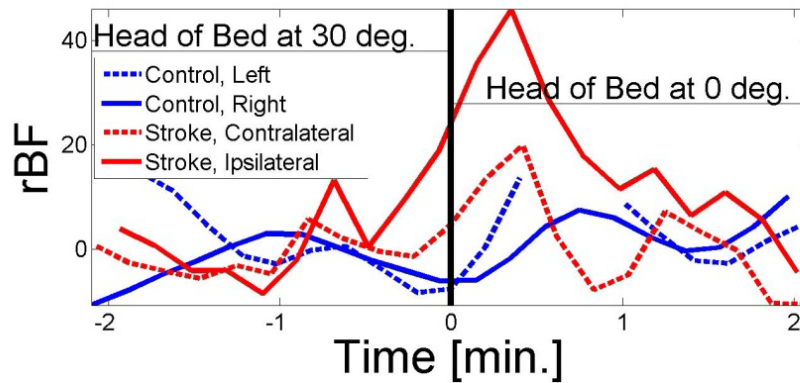


Fig. 1. Relative blood flow (normalized to baseline at 30 deg, rBF) measured by DCS versus time during postural change for Control and Stroke subjects. The head of bed was lowered from 30 deg to 0 deg (flat) at $t = 0$. The stroke patient had a right MCA occlusion, the control had healthy cerebrovasculature. Both subjects were 16 year old males (unpublished data)

In this talk, I will review diffuse optical techniques for both imaging and monitoring, then discuss their application to monitoring cerebral oxygen perfusion in children.

Acknowledgements

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RECONSTRUCTION IN FLUORESCENCE DIFFUSE TOMOGRAPHY BASED ON NON-NEGATIVITY CONDITION

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Abstract: We propose a novel method for solving a system of linear equations based on the non-negativity condition. This method was applied for reconstruction in fluorescence diffuse tomography and was compared with other well-known methods (Tikhonov regularization, ART, SMART, NNLS).

1. Introduction

Fluorescence Diffuse Tomography (FDT) is an emerging technique for 3D imaging of deep-seated fluorescent probes (through several millimeters to centimeters) in biological tissue *in vivo* [1]. In comparison with X-ray and MRI tomography, the reconstruction procedure in FDT and other optical diffuse methods is ill-posed [2]. The ill-posedness is conditioned by high optical scattering in biological tissue. The inverse problem of FDT is usually described by a system of linear algebraic equations (SLAE). Under certain conditions SLAE may have no solution, solution may be non-unique, but the most important thing is that this solution is unstable relative to small deviations of experimental results. To solve such ill-posed problems prior information about solution is generally used. In this work we employ only one obvious "assumption" about non-negativeness of the fluorophore concentration, that enables one to reduce significantly the condition number of the reconstruction problem, thus improving reconstruction accuracy. Despite the fact that this condition is a natural one, little attention has been paid to it in the literature devoted to the FDT reconstruction problem. In this work we present a new method based on the Tikhonov functional that takes into account the non-negativity condition and compare it with the reconstruction techniques mentioned above in model experiment.

2. Methods

The spatial distribution of fluorophore concentration $\Phi(\mathbf{r})$ can be found as a solution of the Fredholm integral equation of the first kind [3]

$$P_f^{\text{exp}}(\mathbf{r}_s, \mathbf{r}_d) = \int_V \Phi(\mathbf{r}_0) F(\mathbf{r}_s, \mathbf{r}_0, \mathbf{r}_d) d\mathbf{r}_0 \quad (1)$$

with the condition $\Phi(\mathbf{r}_0) \geq 0, \forall \mathbf{r}_0 \in V$, where V is the volume within which the fluorophore concentration is reconstructed. Measurements yield a finite number M of the values of function $P_f^{\text{exp}}(\mathbf{r}_s, \mathbf{r}_d)$ which correspond to M different positions of the source and detector pair. Note that we speak about reconstruction of relative rather than absolute values of the distribution function $\Phi(\mathbf{r}_0)$ in volume V because of the large number of multiplicative parameters in the point spread function $F(\mathbf{r}_s, \mathbf{r}_0, \mathbf{r}_d)$.

For numerical solution of the integral equation (1) we divide the initial volume V into N pairwise disjoint sets. The simplest way for discretization of volume V is uniform partitioning of the initial object along the x , y , z -axes into N_x , N_y , and N_z parts, respectively (so that $N = N_x \times N_y \times N_z$) with the corresponding lengths dx , dy , and dz . Therefore, the initial integral equation reduces to a system of linear equations with dimension $M \times N$:

$$Av = p, A \in R^{M \times N}, v \in R^N, p \in R^M, v = \{v_j \geq 0, j = \overline{1, N}\}. \quad (2)$$

We developed an iteration procedure for solving Eq. (2), which is based on the Tikhonov regularization with non-negativity condition (TRNNC) [4]:

$$\begin{aligned} u^{(k+1)} &= (1 - \gamma)u^{(k)} + \gamma(D(u^{(k)})^T A D(u^{(k)}) + \alpha E)^{-1} A^T p \\ v_j^{(k+1)} &= \left(u_j^{(k+1)}\right)^2, j = \overline{1, N} \end{aligned} \quad (3)$$

where α is the regularization parameter, $0 < \gamma < 1$ is the relaxation parameter of the iteration procedure introduced for its robustness, $D(u) = \text{diag}(u)$, E is the unit $N \times N$ matrix.

3. Results and discussions

For testing the developed TRNCC method we conducted a simple model experiment with a medium with homogeneously scattering and absorbing buffer containing a spherical fluorescing inclusion. The optical parameters of the rectangular medium at the excitation $\lambda_{ex} = 532$ nm and emission $\lambda_{em} = 620$ nm wavelengths were $\mu_a(\lambda_{ex}) = 0.02 \text{ mm}^{-1}$, $\mu_a(\lambda_{em}) = 0.005 \text{ mm}^{-1}$, $\mu_s(\lambda_{ex}) = 0.9 \text{ mm}^{-1}$, $\mu_s(\lambda_{em}) = 0.6 \text{ mm}^{-1}$, and $g(\lambda_{em,ex}) = 0.7$. The matrix of SLAE A for this experiment had the following properties: $N = 19228$, $M = 11664$, $s_{\max}(A) \approx 1.4$, $s_{\min}(A) < 10^{-21}$, $m(A) \approx 2 \cdot 10^{-4}$.

Method	Size along XY, mm	Size along XZ, mm	Reconstruction time, s
<i>Initial</i>	2.0	2.0	—
Tikhonov regularization	4.0	9.0	300
ART	4.0	6.7	1500
SMART	2.0	3.5	240
NNLS	1.0	1.0	500
TRNNC	2.0	2.0	2350

One can see from the table that the regularization-based methods (ART [3, 5, 6] and TR [2]) result in blurred initial distribution. SMART [5, 6] allows obtaining distribution close to the initial one, but (due to the exponential convergence of the iteration procedure) the increase of the number of iterations does not improve the quality of reconstruction. NNLS [8] accurately reconstructs only object's center, but not object's size, which is connected with peculiarities of accounting for non-negativity conditions inherent in this method (artificial vanishing of negative components of the solution vector and elimination of the corresponding column of the system matrix at each iteration).

The developed TRNNC method proved to be the best in reconstructing location of the fluorescent inclusion and assessing its size. This method is iterative, but each iteration is based on the results obtained by the direct method (matrix inversion). Thus, the high accuracy of the developed method is provided by Tikhonov regularization, and the non-negativity condition is provided by the iterative procedure which leads to fewer errors in the reconstruction compared with other methods.

Acknowledgements

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METHODS OF CLUTTER REDUCTION IN EPI-ILLUMINATION OPTOCOUSTIC IMAGING

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Abstract. We investigate novel methods which allow clutter elimination in deep optoacoustic imaging. Clutter significantly limits imaging depth in clinical optoacoustic imaging, when irradiation optics and ultrasound detector are integrated in a combined handheld probe for flexible imaging of the human body. Strong optoacoustic transients generated at the irradiation site obscure weak signals from deep inside the tissue, either directly by propagating towards the probe, or via acoustic scattering. We demonstrate that signals of interest can be distinguished from clutter by tagging them at the place of origin. Phantom and first *in-vivo* studies show that clutter can significantly be reduced leading to strongly improved contrast for deep OA imaging.

In optoacoustic (OA) imaging, tissue irradiation using pulsed laser light, and subsequent thermoelastic conversion of absorbed light to ultrasound allows the detection of optically absorbing structures deep inside biological tissue with high resolution using ultrasound receive beamforming. This technique is especially promising for functional imaging of the vasculature, in particular of the oxygenation level based on the different optical absorption spectra of oxy- and deoxyhaemoglobin. In addition, gold nanoparticles, tailored to strongly absorb light in the NIR range, can serve as contrast media, and their functionalization for specific biochemical targets potentially allows early detection of diseases such as cancer and atherosclerosis.

The potential of OA imaging as an additional functional imaging modality augmenting classical ultrasound (US) in a real-time, safe, economical, and versatile multimodal device for improved clinical diagnostics has been demonstrated. For such a combination, an epi-style setup is preferred where the optical components are attached to the acoustic probe for irradiation of the tissue through the same surface where the PA signal is detected. This allows the clinician to guide the combined probe with a single hand. Most importantly, however, the epi-illumination-mode, as opposed to orthogonal or transmission mode, enables imaging of body parts where bones and gas would obstruct propagation of acoustic waves from the illuminated tissue region to the acoustic probe.

An important requirement for a clinically successful combination of OA and US imaging is an adequate imaging depth of several centimeters. Such imaging depths are theoretically predicted as feasible, taking into account the optical attenuation and the front-end electronic noise. Such an imaging depth has, however, been difficult to achieve in practice. An important contributing reason is that the epi-OA setup causes severe clutter, which degrades signal-to-background contrast and therefore limits imaging to a depth considerably less than the theoretically possible noise-limited depth. As a result, an imaging depth of around 1 cm or even less is typically achieved when using 7.5 MHz probes. Clutter can emerge from strong OA transients that are generated at the site of tissue irradiation close to the ultrasound probe, where optical absorbers such as melanin and microvasculature are exposed to the highest laser fluence. These transients travel to the acoustic probe on a direct way, generating direct clutter, but also after being scattered by echogenic structures located inside the tissue, causing echo clutter. Either type of clutter can obscure weak signals from deep inside the tissue. Clinical OA imaging thus requires methods for clutter reduction to achieve the theoretical depth of several centimeters. For this purpose, deformation-compensated averaging (DCA) was previously developed [1-3]. DCA exploits the clutter decorrelation that results from tissue deformation when slightly palpating the tissue with free hand motion of the imaging probe. Motion compensation of the resulting OA images and subsequent averaging maintains true OA detail but reduces decorrelating clutter similar to stochastic noise. DCA benefits from the combination of OA with US because accurate knowledge of tissue motion can directly be obtained from US speckle tracking.

Evaluation of DCA in combined clinical OA and US imaging of human volunteers has demonstrated that although clutter can significantly be reduced, it still has some inherent disadvantages: First, it can only be employed for easily deformable tissue such as the breast, and it requires considerable practice for controlled probe motion. Second, and more importantly, the clutter reduction achievable is limited by the minimum deformation required for clutter decorrelation. As a result the number of im-

ages with independent clutter typically reduces to around ten, allowing for a maximum contrast gain of about three. A significantly larger contrast gain, however, is necessary if the noise limited imaging depth is to be achieved.

To overcome these disadvantages we developed a novel method, localized vibration tagging (LOVIT), which theoretically allows full clutter elimination without the need for tissue palpation. Transient localized tissue vibration “tags” the OA signal at the place of origin, allowing the potentially unambiguous identification of this signal and thus full clutter cancellation. Such localized transient vibration can be induced by means of the acoustic radiation force (ARF) generated by an ultrasonic focused beam. If technically feasible, using the same transducer for both imaging and transmission of the ARF beam will have the strong advantage that the focus of the ARF beam is inherently aligned with the OA imaging plane. Furthermore, the use of a transducer array will allow steering of the focused beam via the transmit phase of the individual transducer elements for flexible generation of ARF in any location within the imaging plane. ARF generation using transmit beam forming is already implemented in radiation force based ultrasound elastography methods, such as acoustic radiation force impulse (ARFI) imaging and shear wave elastography (SWE), where ARF ultrasound transmission over a fraction of a millisecond generates a localized tissue displacement on the order of a few tens of micrometers.

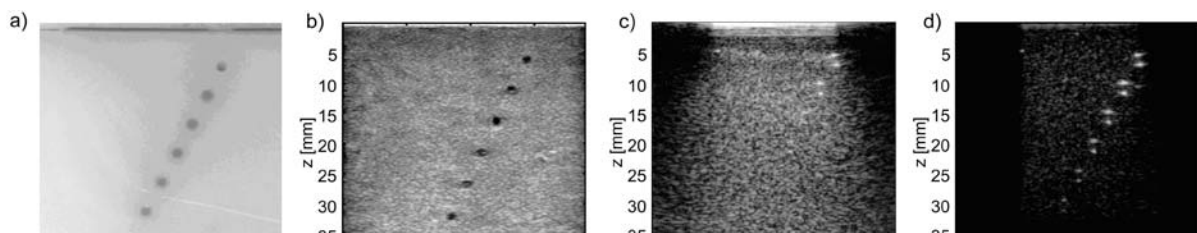


Fig. 1. a) is a phantom section corresponding to the imaging plane showing 2 mm diameter optically absorbing (India ink) and hypoechoic (no cellulose) gelatine cylinders, imitating blood vessels on US in b). c) best OA image without clutter reduction. d) ARF-LOVIT image obtained by mosaicing results from many push focus locations where, at each location, a difference (push minus no-push) image was calculated with normalization to ensure equal noise level for conventional and ARF-LOVIT images

The talk will present our first experimental results as a proof-of-principle of ARF-LOVIT performed on tissue phantoms and on volunteers. We demonstrate for the first time nearly full clutter elimination and virtually noise-limited epi-OA imaging of tissue-mimicking gelatine phantoms. The phantoms contain TiO_2 optical scatterers, cellulose ultrasound scatterers, and India ink for optical absorption. The ARF-LOVIT results are comprehensively compared to classical epi-illumination OA images that were obtained using the same setup. ARF-LOVIT demonstrated greatly improved SNR and imaging depth over conventional OA imaging. Many LOVIT implementations are possible and the method may offer benefits in conventional US and other imaging modalities if clutter limits image contrast.

Acknowledgements

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COMMON PATH CROSS-POLARIZATION SPECTRAL DOMAIN OCT WITH ORTHOGONAL INCOHERENT WAVES

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Abstract. We consider a modification of the common-path spectral domain OCT that allows obtaining cross-sectional images in CO- and ORT-channels when probing a birefringent medium with weak depolarization at scattering as well as cross-sectional images at efficient cross-scattering.

The polarization OCT modalities are supplementary forms of bioimaging that increase informativity of examination by adding qualitatively new information about medium properties. A series of polarization OCT methods have been developed that enable experimental medicobiological studies aimed at revealing specificity of the newly obtained information [1]. Simultaneously, search for most optimal optical CP OCT schemes and operating modes to be implemented in clinical practice is under way. The best informativity of CP OCT systems and reliability of data obtained was demonstrated by CP OCT systems with the use of bulk elements and by diagnostic systems that do not demand flexible change of optical paths during probing. Elaboration of endoscopic OCT modalities faces some difficulties concerned with the use of the available polarization methods in fiber optics. The fiber-optic systems based on anisotropic fiber that allows maintaining the interference signal level during probing under the condition of continuously changing probe deformation played a significant role at a certain stage of OCT techniques development. The influence of deformation of the optical path of a probe wave was eliminated by using a polarization maintaining (PM) fiber and common path optical schemes on isotropic fiber. In addition, the problem of reproducibility of fiber-optic probes was also solved in the common path systems.

Another, no less important aspect of CP OCT is a complex dependence of received signals on the polarization of the initial probe wave and on the orientation of the anisotropy axes of the studied medium [1]. This dependence is most effectively removed either by modulating the polarization state [2-3] or by using two incoherent [4-5] orthogonally oriented waves. One more effect is that in the case of isotropic scattering the backscattered signal received in orthogonal polarization is maximum at circular polarization of the probe wave [6].

The enumerated factors are taken into account in the development of new CP OCT systems. In the presented work we implemented a new polarization-sensitive system based on the common path spectral domain polarization-sensitive OCT (PS OCT).

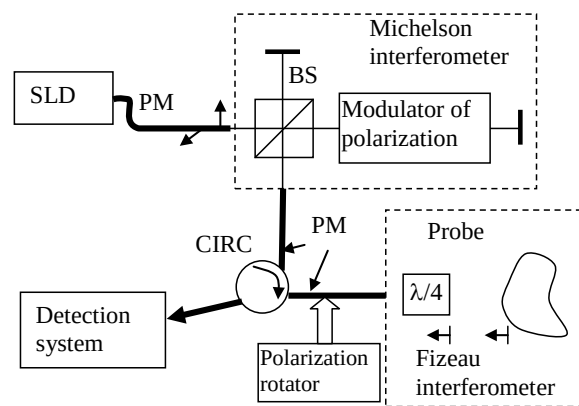


Fig. 1. Optical scheme of common path polarization-sensitive spectral domain OCT

The optical scheme of the new common path spectral domain PS OCT system shown in fig. 1 uses two orthogonal incoherent waves. The optical scheme works as follows. Radiation from the output of a superluminescence diode (SLD) is fed to a PM fiber in equal portions in orthogonal waves. For a

definite length of the PM fiber these linearly polarized waves acquire a delay exceeding the length of the SLD radiation coherence. One of the arms of the compensating Michelson interferometer houses a system controlling polarization state of reference waves. Further, these linearly polarized waves on passing the PM fiber and the $\lambda/4$ plate oriented at an angle of 45° are reflected from the external surface of the plate and form a system of reference waves. Waves from the second arm of the Michelson interferometer make the same path and arrive at the sample now with circular orthogonal polarization.

During reception of the first B-scan the polarization modulator does not change the polarization states of the two reference wave but changes them to orthogonal ones when digitizing the second B-scan.

Several ideas are combined in this system aimed at extracting optimal OCT signals and polarization information and, at the same time, making it a useful endoscopic tool for clinical applications. To achieve this goal

- a common path optical scheme is used that is optimal for the earlier developed flexible optical probes of the type of a forward looking probe, as it provides their reproducibility;
- a two-channel variant of optical scheme with successive reception of the first B-scan with a conventional OCT-image (Co-channel) and the second B-scan with a signal in orthogonal polarization (ORT-channel) has been implemented;
- circular polarization of probe waves is used to eliminate sensitivity to sample orientation in the principal Co-channel;
- two orthogonally polarized incoherent waves allow equalizing the sensitivity of CO- and ORT-channels and simplify the dependence of signals on parameters of birefringence;
- a possibility of introducing an additional polarization rotator to obtain information about orientation of the axes of a birefringent sample using the signal in the ORT-channel is provided;
- modulation of the signal in the ORT-channel due to birefringence is quadratic relative to modulation in the CO-channel, which permits extracting true distribution of birefringence and removes the need to use Hilbert transform.

The features of the acquired images in both CO- and ORT-channels are specified by the formation of definite polarization states of reference waves by means of a two-pass electrically controlled $\lambda/4$ plate and a two-pass 45° Faraday mirror in a compensating Michelson interferometer. This enables obtaining cross-sectional images in CO- and ORT-channels when probing a birefringent medium with weak depolarization at scattering as well as cross-sectional images at efficient cross-scattering.

Acknowledgements

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THE POTENTIAL OF OPTICAL COHERENCE TOMOGRAPHY IN CARDIOLOGY

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Abstract. We describe our experience gained in *ex vivo* studies of cross-polarization OCT (CP OCT) and in the application of intravascular OCT (IV OCT) for diagnosis and control of arterial sclerotic disease. We use intravascular OCT-images acquired on the OCT setup M2 (LightLab Imaging, Inc., USA) in the Republic Scientific Practical Centre “Cardiology” (Minsk, the Republic of Belarus). *Ex vivo* studies were performed using the CP OCT device “OCT 1300-U”. The device was developed at the Institute of Applied Physics (Nizhny Novgorod, Russia) and equipped with forward-looking endoscopic probe with external diameter of 2.7 mm. Collagen polarization properties allow using CP OCT. Fibrous cap and lipid core contrast may be enhanced using CP OCT that measures tissue birefringence.

The optical coherence tomography (OCT) has a great potential in intravascular imaging as an alternative or a supplementary tool to intravascular ultrasound (IV US). We describe our experience gained in *ex vivo* studies of cross-polarization OCT (CP OCT) and in the application of intravascular OCT for diagnosis and control of arterial sclerotic disease. We use intravascular OCT-images acquired on the OCT setup M2 (LightLab Imaging, Inc., USA) in the Republic Scientific Practical Centre “Cardiology” (Minsk, the Republic of Belarus). *Ex vivo* studies were performed using the CP OCT device “OCT 1300-U”. The device was developed at the Institute of Applied Physics (Nizhny Novgorod, Russia) and equipped with forward-looking endoscopic probe with external diameter of 2.7 mm. Collagen polarization properties allow using cross-polarization OCT. Fibrous cap and lipid core contrast may be enhanced using CP OCT that measures tissue birefringence.

The OCT technique is constantly developing and has good prospects for further expansion:

1) *In vivo* diagnosis of “vulnerable” plaque. A thin fibrous cap is a well known feature of atherosclerotic plaque that results in its rupture. The criteria of unstable “vulnerable” plaque susceptible to rupture were set forth by R. Virmani et al.: fibrous cap thickness less than 65 μm ; a small amount of type I collagen and predominance of type III collagen in the cap; high degree inflammation inside the cap. High resolution of OCT allows *in vivo* identification of a thin fibrous cap less than 65 μm (fig. 1).

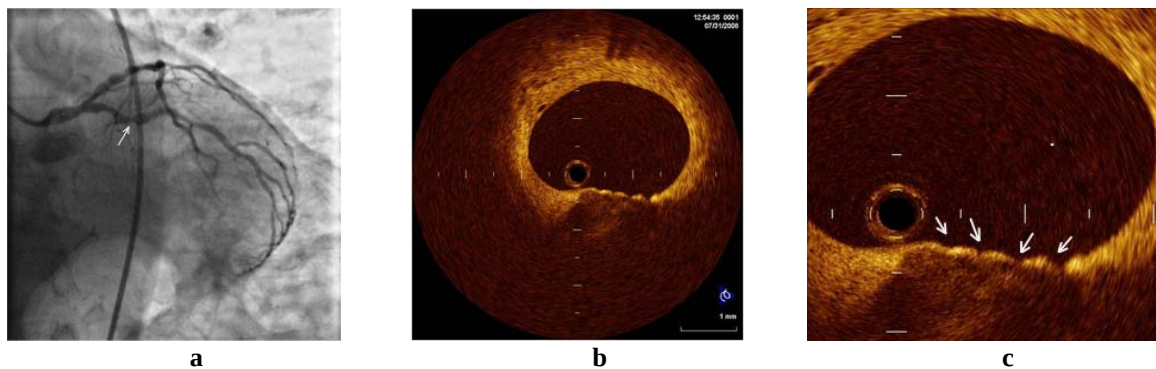


Fig. 1. Unstable atherosclerotic plaque: *a* – coronarography findings: multifocal lesions of coronary arteries, nonuniform staining was detected proximally to stenosis in circumflex branch (arrow) that was suspicious for the presence of recanalized thrombus in this zone; *b, c* – IV OCT is performed proximally to stenosis in circumflex branch (arrow in Fig. *a*); detected: absence of recanalized thrombosis in the studied zone; “incidental” finding: erosion of atherosclerotic plaque cap (arrows indicate erosion zones in Fig. *c*) indicating unstable atherosclerotic plaque

2) Imaging of coronarothrombosis features. Red thrombi are identified as protrusions in arterial cavity with high-intensity signal, whose shadow completely shields the signal. White thrombi are imaged as formations with intense signal that are projected onto the vessel wall and attenuate the signal in this area but to a lesser degree than the red thrombi.

3) Stents follow-up. OCT provides more than US accurate information about hyperplasia rate of neointima on stent struts. OCT is a potentially promising technique when working with stents, as well as in early diagnosis of complications related to this procedure, and long-term follow-up of patients (fig. 2).

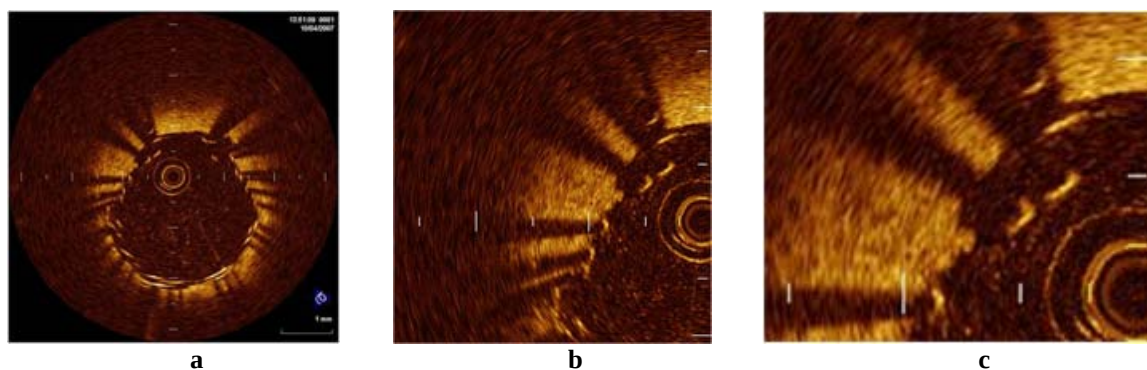


Fig. 2. Poorly outspread stent: *a, b, c* – IV OCT findings with different degree of image magnification: incomplete adhesion at the junction of struts of the stents implanted both to each other and to a vascular wall, indicating incomplete stent apposition in this vascular area (incomplete stent apposition is at 11 o'clock, and at 1–2 o'clock)

4) Separate assessment of plaque elements. OCT enables differentiating between smooth muscle cells and collagen fibers.

Collagen polarization properties allow using CP OCT. CP OCT is a special OCT modality which acquires images resulting from cross-polarization and co-polarization scattering simultaneously. Fibrous cap and lipid core contrast may be enhanced using CP OCT that measures tissue birefringence (Fig. 3).

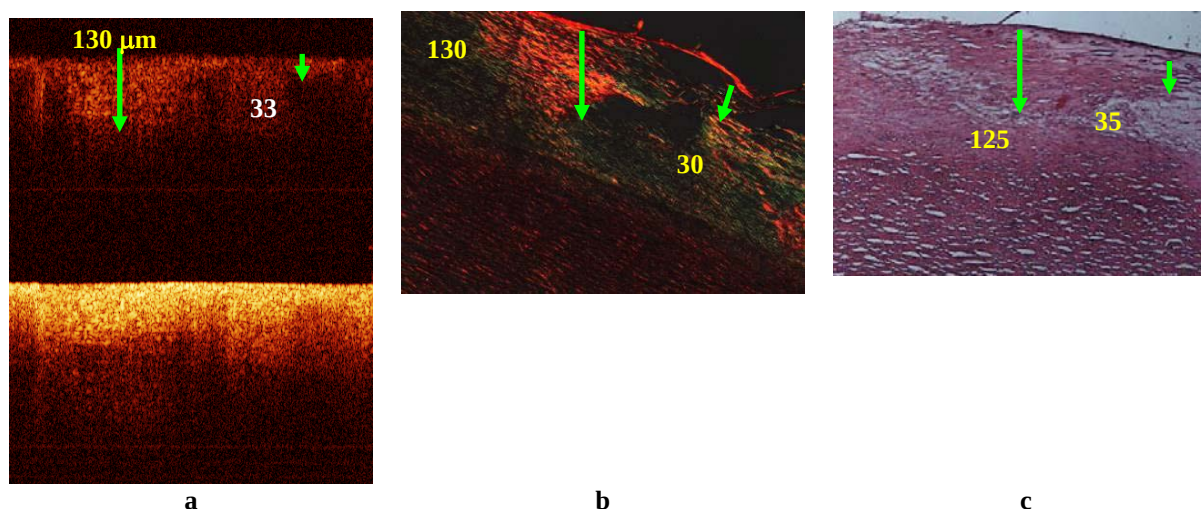


Fig. 3. Unstable atherosclerotic plaque: *a* – CP OCT image in co-polarization (bottom) and in cross-polarization (top); *b* – special histologic picrosirius red (PSR) staining was used with subsequent assessing of the result of collagen staining in polarized light; *c* – H&E staining. The arrows indicated the thickness of the fibrous capsule unstable plaque (in microns)

Acknowledgements

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FAR-RED FRET-SENSOR FOR DETERMINATION OF CASPASE-3 ACTIVITY

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Abstract. Apoptosis is one of the variants of the programmed cell death. Real-time determination of its main effector enzyme - caspase-3 - activity in living cells became possible by means of genetically encoded FRET-sensors. The purpose of our work was to characterize the genetically encoded FRET-sensor based on the far-red fluorescent proteins FusionRed and eqFP670 ("Evrogen", Russia). So, the sensor has the tetrameric structure in solution, and FRET efficiency between donor and acceptor is about 24%. Finally, we showed that our sensor can be used for the caspase-3 activity detection *in vitro* and in living cells by means of the FLIM technique.

Apoptosis is one of the variants of the programmed cell death. The main effector enzyme in the process of apoptosis is caspase-3. Real-time determination of the caspase-3 activity in living cells became possible by means of genetically encoded FRET-sensors.

The purpose of our work was to characterize the genetically encoded FRET-sensor in which the far-red fluorescent proteins FusionRed [1] and eqFP670 [2] were used as a FRET-pair, and these proteins were connected by means of a flexible polypeptide linker containing DEVD tetrapeptide which can be specifically cleaved by caspase-3. The use of far-red fluorescent proteins minimizes the level of background fluorescence and increases the depth of signal penetration.

Dynamic light scattering experiments allowed us to calculate the hydrodynamic radius of a sensor. The result was 6.2 nm, which in the spherical model approach corresponds to the molecular weight of 244 kDa. Thus, as the molecular weight of the sensor is 53.6 kDa, it has the tetrameric structure in solution, similar to the eqFP670 protein.

A decrease of fluorescence intensity of the sensor in comparison with an individual FusionRed protein testifies to the presence of energy transfer between the donor and the acceptor. We also calculated FRET efficiency in the sensor and found that it is about 24%.

Cleavage of our sensor *in vitro* was proved by fluorescence intensity and fluorescence lifetime measurements and by SDS-PAGE.

Finally, we performed experiments on living HEK293T cells expressing our sensor and showed that after induction of apoptosis there is a fluorescence lifetime increase because of sensor's cleavage. So, the designed sensor can be used for determination of caspase-3 activity in living cells.

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ASSESSMENT OF THE "VULNERABLE" ATHEROSCLEROTIC PLAQUE STRUCTURE WITH CROSS-POLARIZATION OPTICAL COHERENCE TOMOGRAPHY (CP-OCT)

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Abstract. We used the CP OCT technique for assessment of collagen in the atherosclerotic plaques (AP). The study was conducted *ex vivo* on 25 post mortem samples of blood vessels of the patients who died from acute coronary heart disease. We demonstrated that CP OCT can distinguish a healthy vessel area from AP. Moreover, it allows differentiating structural components in AP: lipid core, fibrous capsule (FC), areas of calcification. Numerical analysis of the CP OCT images showed that the method permits measuring the thickness of an AP capsule and detect plaques with an FC thickness less than 65 microns (R. Virmani's "vulnerability" criterion) with high accuracy ($p < 0.001$). FC and lipid core contrast may be enhanced using CP OCT that is sensitive to tissue birefringence.

Improved methods are needed to identify "vulnerable" atherosclerotic plaques (AP) which lead to the most acute coronary syndromes. Inflammation plays an important role in the development of atherosclerosis [1]. Inflammatory cells cause collagen breakdown, release of thrombogenic factors, and formation of foam cells. "Vulnerable" plaques, which consist of a thin intimal wall over lipid conglomerates, result in the most acute coronary syndromes through rupture and thrombotic vessel occlusion [2]. The goal of the work was to analyze the AP structure with CP OCT.

Materials and methods. We used the CP OCT technique for assessment of collagen state in AP [3]. Studies were conducted on 25 post mortem samples of blood vessels of the patients who died from acute coronary heart disease. Special histologic picosirius red (PSR) staining of the AP samples with subsequent assessment of collagen staining results in polarized light [4] was used as a control technique for CP-OCT examination.

Results. We demonstrated that CP OCT can distinguish a healthy vessel area from AP. Moreover, it allows differentiating some components in AP: lipid core, FC, areas of calcification. It was shown that it is possible to distinguish the type of collagen in the AP capsule based on OCT signal intensity (Fig. 1).

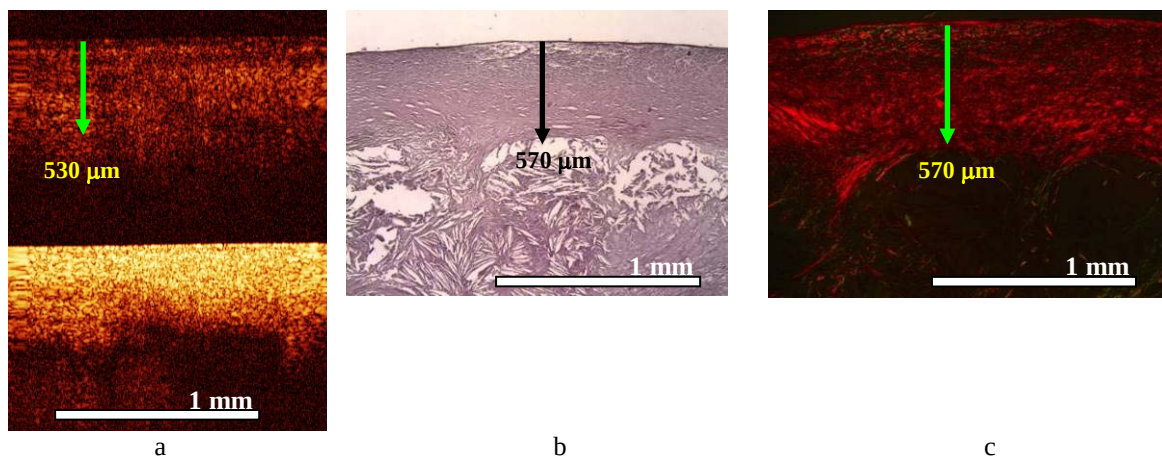


Fig. 1. Stage IV atheroma AP: a – CP OCT image in co-polarization (bottom) and in cross-polarization (top); b – H&E staining; c – PSR staining. The arrows indicate the thickness of the FC plaque (in microns)

Numerical analysis of the cross-polarized OCT images showed that the method allows measuring the thickness of an AP capsule and detect plaques with an FC thickness less than 65 microns (R. Virmani's "vulnerability" criterion) [5] with high accuracy ($p < 0.001$). FC and lipid core contrast may be enhanced using CP OCT which permits assessing birefringent properties of tissue (Fig. 2).

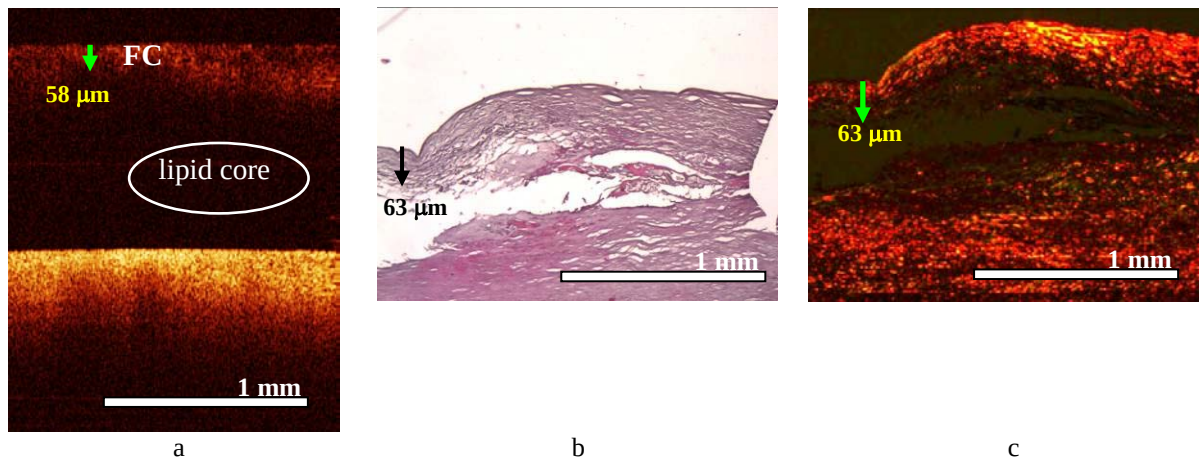


Fig. 2. "Vulnerable" AP, stage Va: a – CP OCT image in co-polarization (bottom) and in cross-polarization (top); b – H&E staining; c – PSR staining. The arrows indicate the thickness of the FC of "vulnerable" plaque (in microns)

In order to exclude subjectivism in assessing CP OCT images of AP we developed automated criteria for high risk plaque recognition. These criteria are based on calculating the integral depolarization factor (IDF) of an OCT signal. The IDF is the averaged ratio of the OCT signal in cross-polarization to that in co-polarization; the ratio is averaged over the pixels where the co-polarization OCT-signal exceeds noise level. The IDF of "vulnerable" AP at stage Va (0.103 ± 0.036) is significantly lower than that of AP at stage IV (0.216 ± 0.081), which have FC thicker than $65 \mu\text{m}$ (Fig. 3). Low IDF of "vulnerable" AP indicates low content of highly organized collagen providing plaque stability.

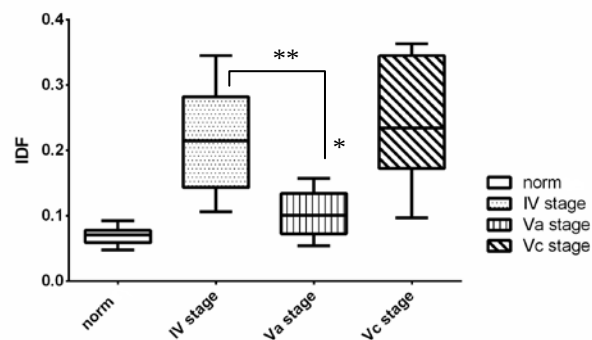


Fig. 3. IDF for CP OCT images of aorta at normal and IV, Va, Vc stages of AP by Virmani. * – statistically significant difference from norm, $p < 0.05$ (one-dimensional Mann -Whitney U-test criterion); ** – statistically significant difference between the specified states, $p < 0.05$ (one-dimensional Mann -Whitney U-test criterion)

Conclusion. The capability of CP-OCT to assess collagen content in a plaque in addition to its high resolution structural imaging makes it a potentially powerful technology for identifying high risk plaques. Analysis of CP OCT images allows assessing the stage of AP and indicates their "vulnerability".

Acknowledgements. The study was carried out under the terms of the Federal Target Program of the Ministry of Education and Science of the Russian Federation "Scientific and academic and teaching staff of innovative Russia" 2009-2013, Agreements No.8145 and 8741; and the grant of the Government of the Russian Federation (No. 14.B25.31.0015).

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MEGAHERTZ OPTICAL COHERENCE TOMOGRAPHY (MHz-OCT): TECHNOLOGY AND APPLICATIONS

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Abstract. We present OCT imaging with line rates in excess of 1 MHz. The two most recent experimental setups are described, at 1050 nm wavelength for retinal imaging, and at 1300 nm for live volumetric video rate imaging. Key to the high imaging speed are Fourier-domain mode locked (FDML) lasers. State of the art FDML technology is discussed. The resulting data stream of >2 Gbytes/sec in the OCT imaging setup can be handled and volume rendered in real time using massively parallel GPU processing. Two different imaging applications are presented showing the potential benefit of such high speed in specific future clinical applications.

Introduction

Optical coherence tomography (OCT) [1] enables the 3-dimensional *in vivo* visualization of tissue micro structure in biomedical imaging applications. Today's fastest commercial OCT systems have imaging speeds of up to 100,000 depth scans per second, most research systems below 200,000. A number of OCT applications in a clinical environment might greatly benefit from even higher imaging speed, but today there are no light sources commercially available for OCT speeds >200 kHz line (A-scan / depth scan) rate. One reason for the limited speed is a fundamental physical limit in standard wavelength swept OCT lasers given by the lasing build up during tuning operation. [2]. Fourier domain mode-locked (FDML) [3] lasers overcome this limit by synchronizing the cavity round trip time to the wavelength tuning repetition rate. FDML lasers have achieved wavelength sweep rates of up to 5.2 MHz [4] and since in OCT one laser wavelength sweep yields one OCT depth scan, FDML lasers enable OCT with >1 MHz line rate. [4, 5]. The image data presented here is used to evaluate the importance of high OCT speed for different future applications.

Experimental setup

For the experiments we used two FDML lasers at 1050 nm and 1300 nm with an optical roundtrip frequency of ~400 kHz. The sweep rate was then multiplied by the so called buffering technique to ~1.6 MHz and ~3.2 MHz. The optical OCT data was digitized with up to 3 Gsamples/s and for real time live video volumetric imaging the data was processed on a Geforce NVIDIA GTX690 dual graphics processing unit. Massively parallel processing on the 3072 CUDA cores can handle the full data rate and enable live volume rendering.

Imaging results and discussion

Figure 1 shows the first application of MHz OCT in ophthalmic imaging at a center wavelength of 1050 nm. 3D volumes consisting of 1080 frames, each consisting of 1080 A-scans with a dense sampling pattern can be acquired in less than a second. Besides standard OCT cross-sections (left) high resolution fundus views (center) and various thickness maps (right) can be extracted from the same single data set in post processing. In clinical applications this can drastically simplify the diagnosis procedure, since the patient will always get a full scan of the entire retina within seconds – all further steps for diagnosis are then performed in post-processing.

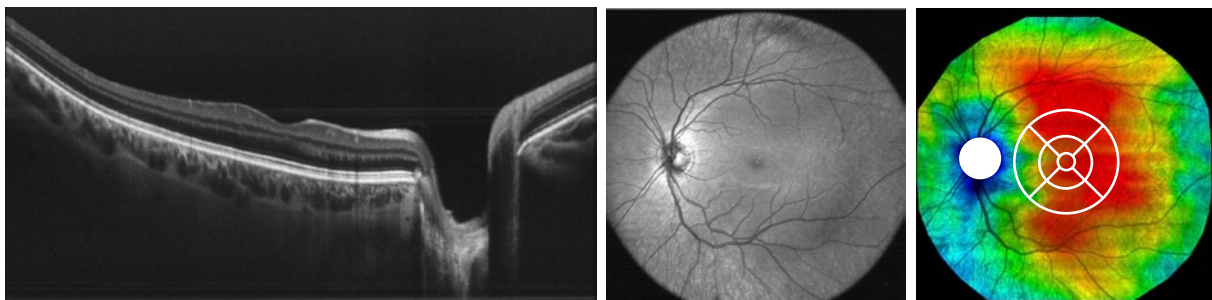


Fig. 1. Application I: Ophthalmic MHz imaging – large, densely sampled volumes can be acquired in less than a second. Beside standard OCT cross-sections (left) high resolution fundus views (center) and various thickness maps (right) can be extracted from the same single data set in post processing

Figure 2 shows the second application of MHz OCT: Live 3D volumetric imaging at video rate. The volumes consist of $320 \times 320 \times 320$ voxels and can be acquired at a rate of 25 Hz. Our current system can also process and volume render such rates live in real time. The example in Fig. 2 shows the observation of a hypodermic needle over human skin *in vivo*. The possibility of using such systems for guiding surgical instruments is evident.

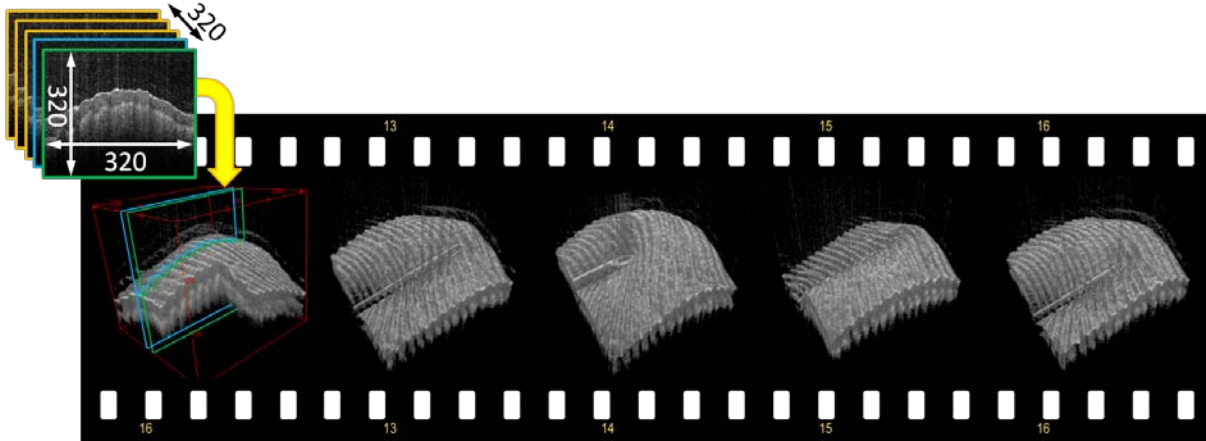


Fig. 2. Application II: Live 3D volumetric imaging. Volumes consisting of $320 \times 320 \times 320$ voxels can be acquired at a volume rate of 25 Hz. The figure shows images from a volume stream observing a hypodermic needle over a human finger tip *in vivo* as a model for surgical guidance applications in the future

Conclusion

Our OCT imaging examples suggest that there are several OCT imaging applications in a clinical environment which might greatly benefit from OCT systems capable of running at MHz A-scans rates.

Acknowledgements

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INVESTIGATION OF THE FLUORESCENCE RESPONSE FROM DEEP-SEATED FLUOROPHORE FOR THE FLUORESCENCE LIFETIME IMAGING OF BIOLOGICAL TISSUES

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Abstract. We investigated the blur of fluorescence kinetics caused by light scattering in biological tissues for different depths of the fluorophore using analytical models of light transport and Monte-Carlo simulation. We have found that the difference between the lifetime estimated by the blurred fluorescence kinetics and the initial lifetime is only several picoseconds for depths up to 2 cm. We also investigated the possibility of estimating the concentration of fluorescent agents having different decay kinetics in the presence of noise, which is necessary for studying FRET *in vivo*.

Fluorescence lifetime imaging is extremely important for a number of biological studies and can be performed in microscopic studies [1] using pulsed picosecond lasers for fluorescence excitation and either time-correlated photon counters or cameras with a gating time of 200 picoseconds or less for the registration of fluorescence decay kinetics (fluorescent agents lifetime is about 1-5 ns). FLIM allows evaluating the ratio between concentrations of fluorescent components having different decay kinetics [2], [3] which is important for studies using FRET [4]. However, it is necessary to take into account the light scattering in biological tissues which blurs the excitation pulse and the fluorescence response as well. This phenomenon is essential for *in vivo* studies. This blurring leads to differences between the measured and initial lifetimes of a fluorescent agent. In order to investigate this effect we used analytical solution [5] of radiative transfer equation (RTE) in diffusion approximation [6] which works for depths >1 mm and Monte-Carlo simulation which gives the solution of RTE for all depths. Fluorescence response from deep-seated fluorophore can be obtained by convolution of the excitation pulse, propagated to a certain depth (position of a fluorophore), initial fluorescence decay kinetics and emission light propagated from fluorophore to the tissue surface.

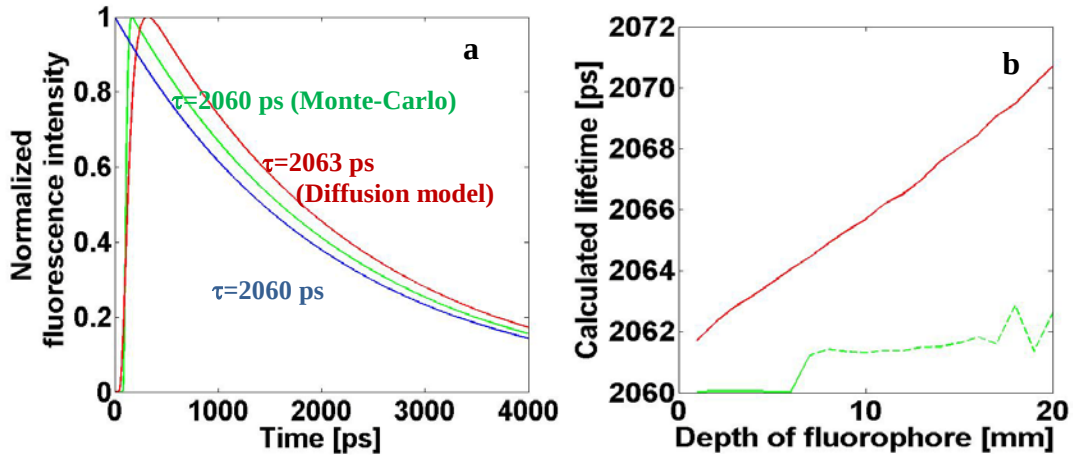


Fig. 1. (a) Normalized fluorescence decay kinetics from fluorophore located at the depth of 5 mm calculated using diffusion approximation of RTE – red line and Monte-Carlo simulation – green line and initial decay kinetics – blue line; (b) dependence of the lifetime calculated by using diffusion approximation of RTE – red line and Monte-Carlo simulation – green line for different fluorophore's occurrence depth

For numerical experiments we took a fluorophore with a lifetime of $\tau = 2060$ ps (which is typical for red fluorescent proteins) in the medium with absorption coefficient $\mu_a^{exc} = 0.1 \text{ mm}^{-1}$, scattering coefficient $\mu_s^{exc} = 4 \text{ mm}^{-1}$ and anisotropy factor $g^{exc} = 0.7$ for excitation light (532 nm) and $\mu_a^{em} = 0.05 \text{ mm}^{-1}$, $\mu_s^{em} = 2.79 \text{ mm}^{-1}$, $g^{em} = 0.7$ for fluorescence emission (625 nm). One can see from Fig. 1 that the effect of blurring leads to an increase of the measured lifetime calculated using different approaches. But the difference between the calculated and initial lifetimes for depths <2 cm is only

several picoseconds, which is close to the precision of the devices applied for FLIM. Moreover, for small depths (<7 mm) the lifetime calculated in diffusion approximation gives larger error than for the Monte-Carlo simulation that gives the error <1 ps. For this region of depths, the results obtained by Monte-Carlo simulation are more reliable than for the diffusion approximation. For depths >1 cm the results of Monte-Carlo simulation look suspicious (dashed line); these results will be recalculated using larger photon statistics. Nevertheless, the blurring effect for depths <2 cm seems to be insignificant in comparison with noise distortion of fluorescence decay kinetics which must be taken into consideration is assessing the ratio of the concentrations.

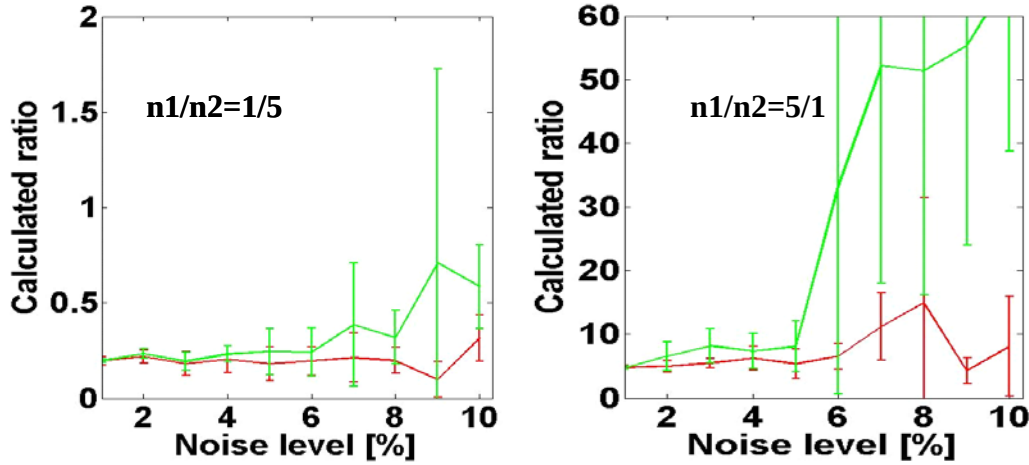


Fig. 2. Calculated ratio of concentrations versus noise level.
Single exponential model – green line, double exponential model – red line

Evaluation of the ratio between the concentrations of the fluorescent components having different decay kinetics is traditionally provided by approximation of fluorescence kinetics with a single exponential model $S = S_0 e^{-\frac{t}{\tau}}$ and the concentrations ratio is obtained by the calibration curve. This method works well in low noise conditions in microscopic investigations. For *in vivo* imaging the signal-to-noise ratio can be significantly less and estimation of the concentrations ratio provided by this method gives a large error (Fig. 2). We investigated the stability of the double exponential method which uses

the approximation $S = N_1 e^{-\frac{t}{\tau_1}} + N_2 e^{-\frac{t}{\tau_2}}$ instead of a single exponential one. In our numerical experiment the ratio of concentrations in the mixture of fluorescent agents with $\tau_1 = 2060$ ps and $\tau_2 = 3150$ ps (the real ratio was 1/5 and 5/1) in the conditions of additive Gaussian noise of different dispersion. As one can see from Fig. 2, the double exponential model is more stable to the presence of noise in comparison with the single exponential model.

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INTERPRETATION OF OCT IMAGES IN BIOTISSUE DIAGNOSTICS: NUMERICAL SIMULATION AND ANALYSIS

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Abstract. OCT-images of biotissues in norm and pathology often require interpretation from a clinician who is not familiar with physics behind OCT-image formation. We present numerical simulation of OCT-image by means of Monte Carlo technique as a tool for understanding the principles of formation of OCT-images in dermatology. Numerical analysis of OCT-images of biotissue in norm and pathology can help to exclude clinician's subjectivism in their interpretation. We demonstrate application of histogram analysis for recognition of fallopian tubes pathology in OCT-laparoscopy and calculation of integral depolarization factor in evaluation of OCT-images of bladder and arterial walls.

Optical coherence tomography (OCT) is actively penetrating into clinical practice beyond ophthalmology [1]. However, OCT-images of biotissues in norm and pathology often require interpretation from a clinician who is usually not familiar with physics behind OCT-image formation. In such situation numerical simulation of OCT images can help in understanding of OCT-image formation of biotissues with different structure and optical properties and predict the effect of different factors, such as compression or application of contrasting agents, in OCT diagnostics. In this paper we demonstrated application of Monte Carlo simulation of OCT-images for interpretation in OCT-dermatoscopy and development of techniques for OCT-image contrast enhancement.

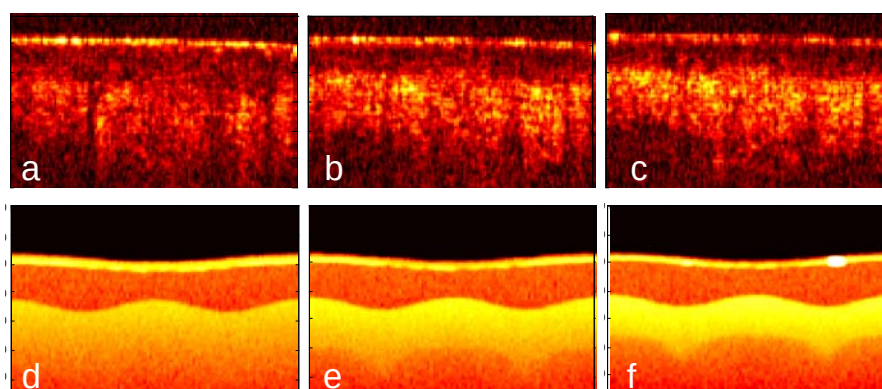


Fig. 1. Experimental (a-c) and Monte Carlo simulated (d-f) OCT-images of human skin immediately (a, d), in 3 minutes (b, e), and in 6 minutes (c, f) after compression start

Monte Carlo statistical technique of OCT is based on simulation of a large number of random photon trajectories in predefined medium configuration and construction of OCT-image basing on detection conditions [2]. In this work we apply Monte Carlo simulation in study of mechanism of human skin OCT-image contrasting under mechanical compression. This effect is based on different elastic properties of skin layers and physiologic response [3]. Qualitative agreement of experiment and simulated OCT-images in course of compression allows to evaluate induced changes in properties of human skin layers (Fig. 1). Simulations were also applied in OCT study of difference in structure of thick and thin human skin.

Another approach which can help avoiding subjectivism in evaluation of OCT-images in diagnostic process is their numerical analysis. We employed such analysis for development of algorithm for automated recognition of pathologic states in OCT-laparoscopy. OCT-laparoscopy is an OCT inspection of fallopian tubes during standard laparoscopic procedure aimed for revealing the reasons of infertility in female patients [4]. In this clinical study 176 patients were enrolled which allowed distinguishing norm and two types of pathology: edema and fibrosis in OCT-images (Fig. 2, a–c). Typical features

allowed to develop two scores; one of them is based on histogram analysis (S_1) and allows to separate norm from fibrosis, while another is based on 1st derivative of the in-depth OCT-signal (S_2) and allows to separate norm from edema (Fig. 2, d).

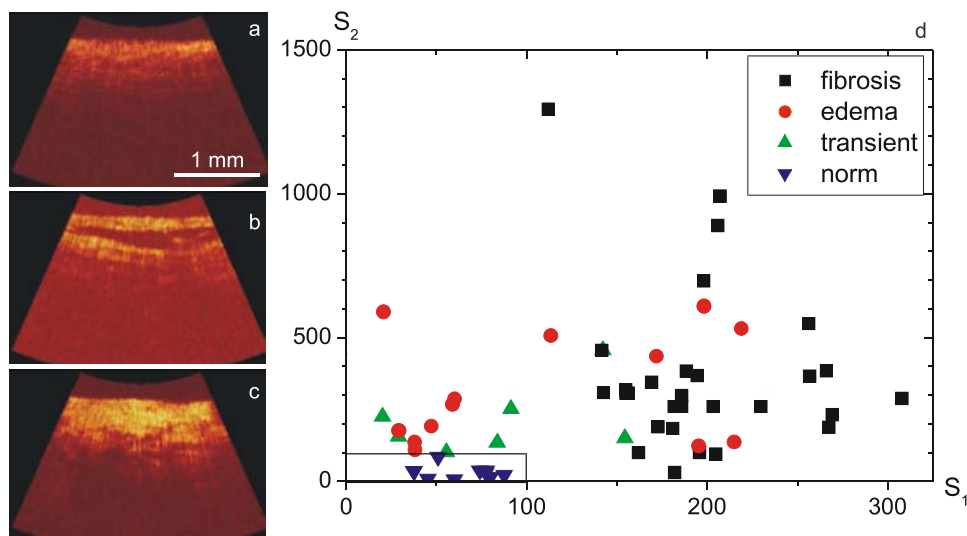


Fig. 2. OCT-images of fallopian tube tissue in vivo: norm (a), pathology with prevalence of edema (b), and fibrosis (c). Characterization of norm and pathology OCT images by independent criteria scores for reference (a) and test (b) groups. Rectangular zone limits score values for norm

Introduction of these scores allowed to increase diagnostic efficacy of OCT-laparoscopy: under subjective recognition the diagnostic characteristics are flowing: sensitivity 90%, specificity 81%; diagnostic accuracy 88% while introduction of independent criteria allowed to additionally increase them to the following values: sensitivity 96%, specificity 100%; and diagnostic accuracy 96%. In perspective these criteria can be employed for automatic recognition of OCT images and processing of video-stream.

Polarization sensitive OCT (PS OCT) is a promising tool to estimate the depolarization properties of the imaged biotissue that are typically attributed to the presence of collagen. However, adequate assessment of collagen state (content and structure) from PS OCT images requires simultaneous analysis of both OCT image corresponding to the initial polarization and the image corresponding to the orthogonal one. We have developed a numerical procedure which results in evaluation of the integral depolarization factor (IDF), a quantitative dimensionless parameter related to the depolarization ability of the imaged biotissue and insensitive to the OCT setup configuration and the probing beam retardation. IDF calculation approach has been tested on biotissie samples of bladder and arterial walls in norm and pathology. Using independent methods of collagen state estimation (polarization microscopy with Picrosirius Red staining, immunohistological analysis) we have shown that the change of IDF value is in perfect correlation with the change of collagen state in biotissue.

Acknowledgements

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CP OCT ASSESSMENT OF THE DEPOLARIZING PROPERTIES OF CONNECTIVE TISSUE STROMA IN HUMAN MUCOSA IN VIVO

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Abstract. We used the CP OCT technique for assessment of collagen state of connective tissue stroma in human mucosa. We show that strictly ordered collagen fibers structures are clearly manifested in cross-polarized OCT-images. For solution of the clinical problem of distinguishing collagen states in inflammation, its outcomes and neoplasia *in vivo*, we propose some criteria, such as the integral depolarization factor (IDF) and fractional OCT signal standard deviation (SD_f) calculated from the co- and cross-polarized OCT-images. It was shown that IDF demonstrates higher sensitivity in comparison with SD_f and assesses the depolarizing properties of collagen fibers with different nature of pathology with statistically significant difference ($p < 0.05$).

Cross-polarization OCT (CP OCT) is an OCT modality which acquires images in co-polarization and cross-polarization simultaneously, thereby providing additional information about polarization properties of the inspected tissues such as mucosa membranes [1]. However, evaluation of tissue state from CP OCT images is currently based on visual criteria that are not objective due to effects of instrumental and speckle noise hindering assay of depolarization properties of collagen [2]. **The goal** of the study was to develop numerical criteria for characterization of depolarizing properties of connective tissue stroma in human mucosa with CP OCT in norm and under various pathophysiological conditions.

Materials and methods. The CP OCT studies were carried out with the "OCT 1300-U" device (IAP RAS, Nizhny Novgorod, Russia) [3]. The objects under study were the following: 1) human cheek mucosa; 2) human urinary bladder mucosa during *in vivo* endoscopic OCT manual observation; 3) removed fragments of the mucous membranes from the sites of CP OCT inspection in the course of surgery and biopsy for histological examination. The CP OCT images were compared with the morphological images. We studied the histological specimens of the same tissue by polarizing microscopy with picosirius red (PSR) staining for evaluation of collagen orientation and content. Quantitative analysis of 54 CP OCT images of the oral mucosa and 96 CP OCT images of the bladder mucosa was done to calculate fractional OCT signal standard deviation (SD_f) and integral depolarization factor (IDF).

Results. We obtained CP OCT images from 150 zones of oral and bladder mucosa in norm and under various pathophysiological conditions. Parallel histo-tomographic comparisons and quantitative assessment of CP OCT images and PSR stained images in polarized light were done. Based on clinical and morphological data all the images were divided into the following groups. 4 groups in oral mucosa: 1 – norm, 2 – acute inflammation, 3 – reparative fibrosis formed during chronic inflammation, 4 – sclerosis formed as an outcome of granulomatous inflammation. 7 groups in bladder mucosa: 1 – norm, 2 – acute inflammation, 3 – reparative fibrosis formed during chronic inflammation, 4 – sclerosis formed after injury (scar tissue), 5 – cancer in situ, 6 – flat carcinoma at the early stage of invasion, and 7 – recurrent cancer in scar tissue. Two dimensionless parameters were developed for assessment of cross-polarized OCT images: SD_f which is the ratio of the OCT signal SD in the cross-polarized image to the OCT signal SD in co-polarized image with deduction of OCT signal SD noise level, that was used as a degree of OCT signal brightness in the image; and IDF which is the averaged ratio of the OCT signal in the cross-polarization to that in the co-polarization, that is averaged over those pixels where the co-polarization OCT-signal exceeds noise level.

To establish correlation between OCT signal in cross-polarized image and collagen fibers content in norm, acute inflammation and fibrosis/sclerosis processes we measured brightness in PSR stained images of histological specimens in 4 groups of patients. The results of normalized brightness of PSR stained histological patterns are listed in Fig. 1, a. We can conclude that acute inflammation (group 2) leads to disorganization of collagen fibers and PSR brightness dramatically drops against the norm level, whereas excessive production of collagen fibers and their packing result in an increase of PSR

brightness, depending on intensity of the fibrosis. The same results were obtained by calculating SD_f and IDF. However, similarly to PSR brightness, IDF can establish the difference between slight fibrosis and its expressed form like sclerosis. High correlation between IDF/OCT signal SD_f ($r = 0.84/0.74$, $p = 0.0001$) and normalized brightness of PSR stained collagen fibers in cheek mucosa specimens was found in groups of patients with different content and condition of collagen fibers in stroma of cheek mucosa.

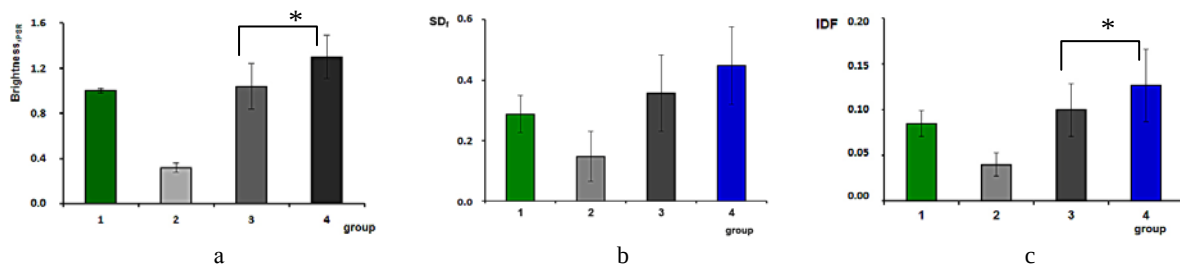


Fig. 1. Results of quantitative assessment of images in various pathophysiological conditions of oral mucosa in cheek area: a – normalized brightness of collagen fibers in PSR specimens of cheek mucosa; b – SD_f of CP OCT images; c – IDF of CP OCT images. * – statistically significant difference between the specified states (Mann – Whitney U-test criterion, $p < 0.05$)

Figure 2 demonstrates the results of SD_f and IFD calculation in groups of bladder mucosa with different pathologies. It is shown that in the groups of acute inflammation (group 2) and fibrosis (group 4) (accompanied by substantial reorganization of collagen fibers) statistically significant differences of SD_f and IFD from the norm ($p < 0.05$) were obtained. We also found a statistically significant difference ($p < 0.05$) of SD_f and IFD mean in groups of chronic inflammation in remission with severe epithelial dysplasia (group 5) and acute inflammation (group 2) as compared to flat epithelial cancer without deep invasion (group 6), and between groups of recurrent cancer in the scar tissue (group 7) and scar tissue (group 4).

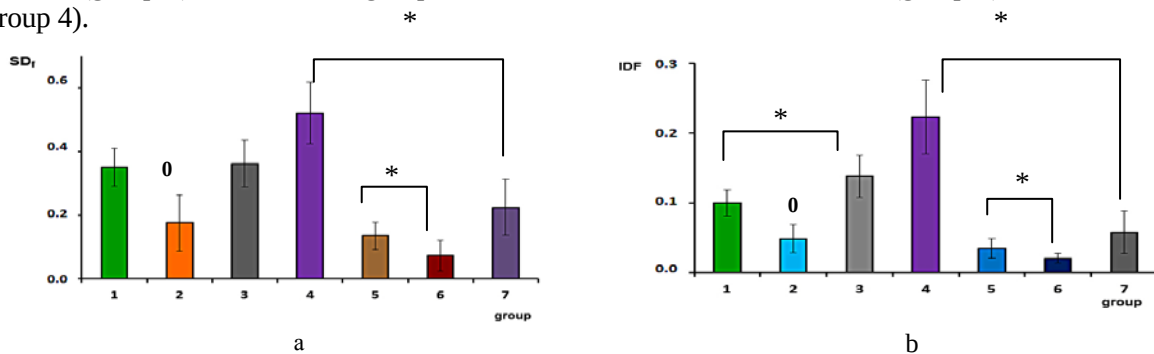


Fig. 2. Results of quantitative assessment of CP OCT images in various pathophysiological conditions of bladder mucosa: a – SD_f ; b – IDF. * — statistically significant difference between the specified states, 0 – statistically significant difference between group 2 and 6 (Mann – Whitney U-test criterion, $p < 0.05$)

The obtained results prove the correlation between the SD_f /IFD and collagen condition in mucous stroma. But IFD recognizes slight fibrosis (group 3) from norm (group 1) better. This fact indicates that IFD is more sensitive to changes of collagen fibers depolarization and has a potential to be used in clinical diagnostics.

Conclusion. The study and quantitative assessment of CP OCT images confirm the presence of depolarizing properties of connective tissue stroma in human mucosa that are determined by well-defined spatial and structural organization of collagen matrix. Collagen fibers dramatically change in pathological processes that affect the ability of its depolarizing properties which can be detected *in vivo* with CP OCT. CP OCT enables differentiating clinically significant conditions of oral and bladder mucosa *in vivo*.

Acknowledgements. The study was carried out under the terms of the Federal Target Program of the Ministry of Education and Science of the Russian Federation “Scientific and academic and teaching staff of innovative Russia” 2009-2013, Agreement No.8145; and the grant of the Government of the Russian Federation (No.14.B25.31.0015).

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IN VIVO BIOLUMINESCENCE IMAGING OF TUMOR CELLS USING FIREFLY LUCIFERASE LUC2

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Abstract. Optical imaging techniques have been utilized as a high-throughput tool for cancer research. One of the most discussed issues of *in vivo* imaging is how to enhance the sensitivity of this method. This work is aimed at developing a luciferine-luciferase system for detection of small tumor cell populations in mice by bioluminescence whole-body imaging. We created a lentiviral vector containing enhanced firefly luciferase gene (*luc2*) and transfected cancer cells. Applicability of the system for *in vivo* imaging of primary tumors and metastases in mice was shown. We demonstrated much higher efficiency of *luc2*-based bioluminescence imaging compared to fluorescence imaging with GFP-like proteins.

Introduction

Optical imaging is an irreplaceable tool to assess dissemination of tumor cells in the body of mice. Although various types of reporter genes encoding fluorescent proteins have been described, expression of exogenous luciferase combined to systemic administration of its substrate remains the strategy of optical imaging providing the best signal/background ratio [1]. The best studied and the most applicable bioluminescent marker appears is the firefly luciferase isolated from *Photinuspyralis*. In the presence of adenosine triphosphate and oxygen, this enzyme oxidizes the heterocyclic substrate D-luciferin to oxyluciferin and emits light in the range from yellow-green to red. The best and brightest firefly luciferase construct for deep tissue *in vivo* bioluminescence imaging is *luc2*. This enhanced luciferase is codon optimized for mammalian cell cytoplasmic expression. *Luc2* emission spectrum peaks at 612 nm in mammalian cells at 37 °C [2]. Bioluminescence imaging based on *luc2* has distinct advantages over traditional approaches in monitoring both primary tumors and metastases in animal models. The goal of this study was to develop the luciferine-luciferase system for detection of primary tumors and metastases at the early stages in mice by bioluminescence whole-body imaging.

Materials and methods

pLuc2-N vector and lentiviral vector pLVT-1 were obtained from the Laboratory of Molecular Technologies (IBCh RAS, Russia). Mouse colon adenocarcinoma 26 (Colo 26) cell line and BALB/c mice were used. We applied the genetic engineering techniques, the lentiviral transduction protocol, the method of bioluminescence imaging, and the histological analysis. Luciferase activity of Colo 26-*luc2* cells was measured *in vitro* and *in vivo* using an IVIS Spectrum (Caliper Life Sciences, USA). Total flux (photons/sec) was quantified using Living Image software 4.2. Serial dilutions of Colo 26-*luc2* cells at an initial concentration of 25 000 cells/well were performed for *in vitro* assay. D-luciferin was added at 150 µg/ml. Colo 26-*luc2* cells in the amount of 25, 50, 100 and 200 were implanted subcutaneously into the mice. The dose of D-luciferin for *in vivo* imaging was 150 mg/kg. The primary tumor model was generated by subcutaneous injection of 500 000 Colo 26-*luc2* cells in mice. The metastases model was created by intravenous injection of 75 000 Colo 26-*luc2* cells in mice.

Results

A lentiviral vector containing the firefly *luc2* gene was constructed to generate a stable bioluminescent cancer cell line. Colo 26 cells were then transfected with this lentiviral vector and a stable Colo 26-*luc2* cell line was created as a result. Colo 26-*luc2* cells were prepared using a serial dilution method for the assessment of the sensitivity of *in vitro* bioluminescence imaging. It was shown that the intensity of bioluminescent signal correlates with the tumor cell number. Total flux accounted for about 5000 photons/sec per cell. Then we proceeded to the subcutaneous injection step to assess the sensitivity of *in vivo* bioluminescence imaging. Different numbers of Colo 26-*luc2* cells were implanted subcutaneously into BALB/c mice. *In vivo* imaging indicated that the minimum number of cells with subcutaneous localization we could detect

was about 50. Total flux of 50 tumor cells accounted for about 9600 photons/sec, 100 cells – 15100 photons/sec, 200 cells – 80000 photons/sec, which was 250 photons/sec per cell on the average. Because Colo 26-luc2 cells showed extremely high light emission *in vivo* we next attempted to visualize the primary tumor at early stages. The ability of Colo 26-luc2 cells to form a tumor node was previously tested. We detected the tumor node on the 4th day after injection of Colo 26-luc2 using *in vivo* bioluminescence imaging. Total flux from the tumor was proportional to the number of live cells expressing luc2 during monitoring of tumor growth. The next step was to examine the metastatic properties of Colo 26-luc2 cells. The mice were implanted intravenously with the cells. On the 9th day after injection intensive bioluminescent signals were detected in the thorax area that corresponded to lung metastases. To confirm metastases of Colo 26-luc2 cells into lungs we isolated lung tissues and took *ex vivo* images. In addition, we performed histological analysis on formalin-preserved, paraffin sectioned tissues. The results showed that the lungs were infiltrated with small groups of poorly differentiated neoplastic cells.

Discussion

Luciferase-labeled cancer cells are widely accepted in mice models of primary tumors and metastases [3]. Although many tumor cell lines expressing bioluminescent marker luc2 such as 4T1-luc2 and PC-3M-luc2 are commercially available, it is important to expand the collection. The present work resulted in creation of a stable Colo 26-luc2 cell line. We used the lentiviral technology for stable cell line generation. Lentiviral vectors open exciting perspectives owing to their ability to govern efficient delivery, integration, and long-term expression of transgenes into cells both *in vitro* and *in vivo* [4]. *In vitro* assessment of Colo 26-luc2 luciferase activity showed that cells responded adequately to D-luciferin adding. The sensitivity of *in vivo* bioluminescence imaging proved to be very high. It allowed detecting a small number of tumor cells with subcutaneous localization (about 50). For comparison, the minimum number of cells labeled with red fluorescent protein TurboFP635 accounts for 90 000 using fluorescence imaging (experimental data). The primary tumor model based on Colo 26-luc2 demonstrates the ability of tumor node detection at early stages. Moreover, a bioluminescent signal correlates directly with tumor size during monitoring and indicates only live cells in contrast to traditional caliper measurements [5]. The Colo 26-luc2 cell line was also tested for early metastases detection in mice. It was found that micrometastases are identified in lungs by whole-body bioluminescence imaging on the 9th day after injection of tumor cells in contrast to the standard 21st day [6]. The studies carried out on Colo 26 cells labeled with red fluorescent protein KillerRed showed the difficulty of metastases detection even at late stages (unpublished data). Thereby bioluminescence imaging based on Colo 26-luc2 cell line proved to be a very sensitive and effective approach for tumor cells monitoring in living mice.

Acknowledgements

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VERSATILE SYSTEM FOR SMALL ANIMAL FLUORESCENCE IMAGING

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Abstract. We present a multifunctional system for small animal fluorescence imaging which combines planar epi-illumination and trans-illumination geometries. We have demonstrated that the created system allows obtaining 2D fluorescent images for estimating the cross-sectional size of the superficial fluorescent objects and deep seated fluorescing inclusion by using trans-illumination imaging even in the case of low fluorescent intensity. We also have shown that for high fluorescence signal the developed system provides 3D reconstruction of the fluorophore spatial distribution. This technique is based on the spectrally resolved measurements provided by synchronous scanning of an animal by a laser source and a spectrometer.

Introduction

Today fluorescence imaging has become an important tool in preclinical drug studies on small animals [1]. There are two basic geometries of the whole-body fluorescence imaging systems: epi-illumination and trans-illumination. Epi-illumination systems [2] allow rapid (1-5 sec) estimation of the fluorescing tumor cross-sectional size. The accuracy of such estimation is higher when the tumor is closer to the surface. Trans-illumination imaging systems [3] require a longer time (a few minutes) to acquire an image. In contrast to epi-illumination technique, trans-illumination imaging is more sensitive to deep seated tumors. Both imaging geometries form the basis of fluorescence diffuse tomography (FDT) systems [4]. The FDT technique enables 3D imaging of the fluorophore distribution. But the image reconstruction is a time-consuming process which requires sophisticated computational algorithms. Each of the fluorescence imaging techniques is widely used in biomedical research for specific tasks.

We have created a versatile system for small animal fluorescence imaging which combines planar epi-illumination and trans-illumination geometries and provides 3D image reconstruction. The scheme of the developed system is shown in Fig. 1. A cooled CCD with a LED-illumination board (illumination wavelengths: 467 nm, 518 nm, 590 nm, 635 nm) are used for fluorescence imaging in the epi-illumination configuration. For fluorescence separation, a filter set is installed between the objective and the CCD. Synchronous scanning of the animal by a laser source of 593 nm for excitation of red fluorescent proteins and a high sensitive cooled PMT (H7422-20, Hamamatsu Co.) allows implementing the trans-illumination imaging technique. Synchronous scanning of the animal by the laser source and the spectrometer (QE65000, Ocean Optics Inc.) is used for the FDT technique.

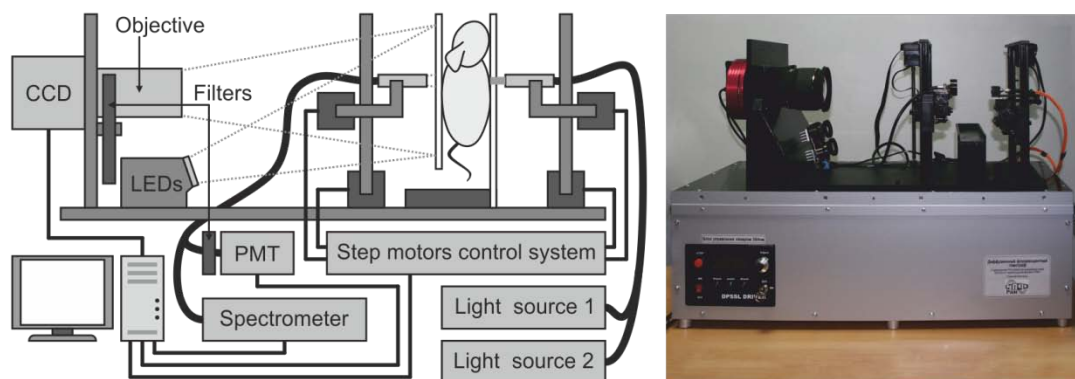


Fig. 1. Versatile system for small animal fluorescence imaging

In vivo monitoring of tumor growth

For testing the created system, monitoring of tumor growth in small animal was conducted using epi-illumination and trans-illumination geometries. Figure 2 shows the growth of subcutaneous HeLa tumor in an immunodeficient nude mouse. The tumor cells were transfected with red fluorescent protein KillerRed [5]. The obtained results showed precision sizing of subcutaneous tumors by the epi-illumination imaging and high sensitivity of trans-illumination imaging for detecting deep seated tumors.

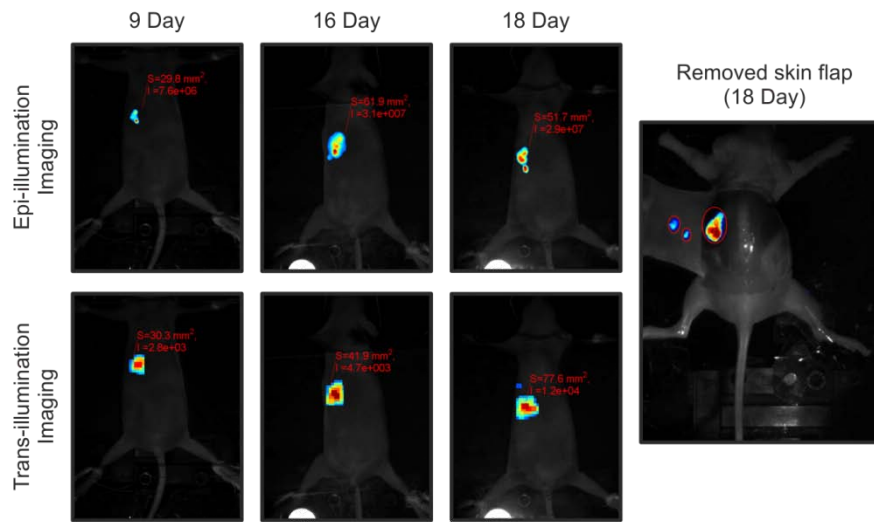


Fig. 2. *In vivo* monitoring of tumor growth

Reconstruction of the fluorophore spatial distribution

The developed system also provides 3D reconstruction of the fluorophore spatial distribution inside animal body based on the spectrally resolved measurements. Advantages of spectrally-resolved FDT over conventional FDT are high accuracy in solving the tomography inverse problem and reducing the biotissue autofluorescence [6, 7]. The results of the experimental studies on small animals *in vivo* using the spectrally-resolved FDT are presented in Fig. 3.

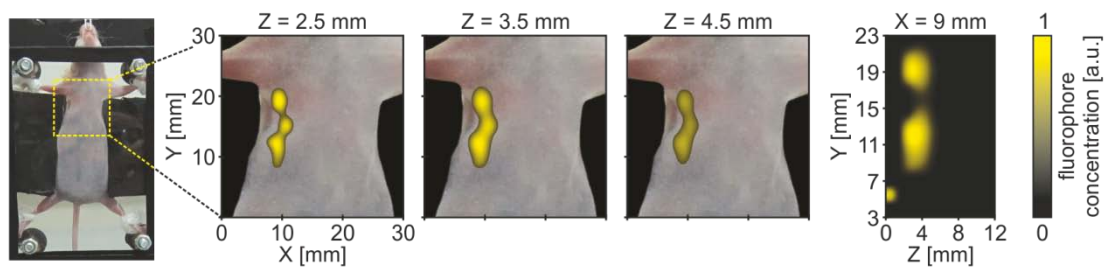


Fig. 3. Reconstruction of the fluorophore spatial distribution inside experimental mouse body

Acknowledgements

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COMPREHENSIVE STUDY OF RADIATION DAMAGE OF EXTRACELLULAR MATRIX OF BIOLOGICAL TISSUE

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Abstract. Molecular mechanisms of radiation damage and regeneration of connective tissue structures of internal organs were studied. Radiation-induced changes of collagen of rat bladder and rectum have been investigated at different times after irradiation with doses of 2 to 40 Gy using complex methods. Irradiation resulted in an increase of collagen denaturation temperature with possible connective tissue hyperplasia, but the general architectonics of bladder and rectum was not altered, independent of the dose and time after irradiation.

Introduction

Radiotherapy, like all other cancer treatment modalities, not only results in beneficial effects with regard to tumor control, but is also associated with side effects in disease-free normal tissues. Organs that are frequently affected by pelvic radiotherapy are the urinary bladder and the rectum, as they are included in the treatment field of a wide variety of tumors, namely malignancies of prostate, uterus/cervix. Connective tissue reaction plays an important role in radiation damage, however, a comprehensive study of the processes of collagen damage and remodeling has not been done until now.

Materials and methods

Rat bladder and rectum were irradiated *in vivo* under general anesthesia (Zoletyl 50 mg/kg) with a ⁶⁰Co external beam therapy unit in order to study the progression of extracellular matrix damage as the gamma dose increased from 2 to 40 Gy. One day, one week and one month after irradiation, the bladder and rectum samples were investigated.

The damage at the molecular and microfibril levels was assessed using differential scanning calorimetry (DSC). The thermal analysis was carried out in DSC20 and DSC30 cells of METTLER TA 4000 calorimeter. Heating was performed up to 95°C with the rate of 10 K/min. Changes at the fibril and bundle level were evaluated using laser scanning confocal microscopy (LSM 510 Meta (Carl Zeiss)) with second harmonic generation (SHG) imaging. Polarization sensitive optical coherence tomography (PS-OCT) was used for assessing changes of general organs architectonics. PS-OCT imaging was done using the OCT device developed at the Institute of Applied Physics of the Russian Academy of Sciences (Nizhny Novgorod). Histological study of rectum and bladder samples was chosen as a method of verification of the results obtained by optical and physico-chemical methods. Specific staining, such as toluidine blue, Van Gieson's picrofuchsin, PAS reaction that allow obtaining information about connective tissue was used.

The calorimetric measurements showed a slight increase of collagen denaturation temperature and extension of denaturation peak in the rectum and in the bladder after irradiation with doses from 2 to 40 Gy (Fig. 1).

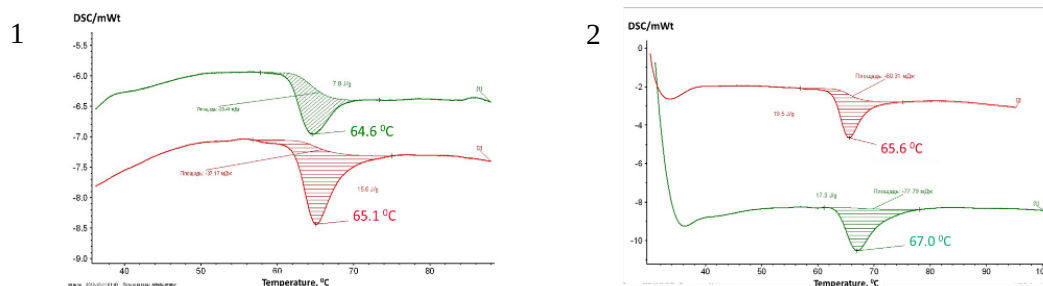


Fig. 1. Typical DSC-thermograms of rat bladder samples: 1 – intact; 2 – 10 Gy, one week

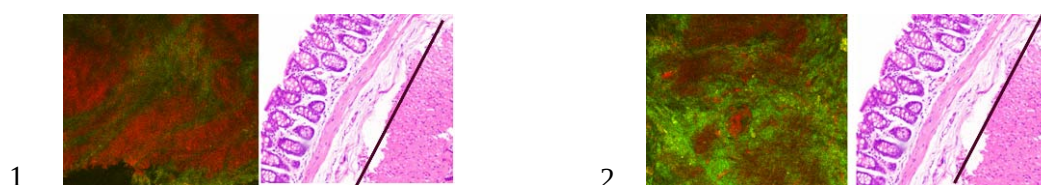


Fig. 2. SHG-images of rat rectum samples (and histological sample with cut level):
1 – intact; 2 – 10 Gy, one week

Substantial changes were detected in the SHG images of the irradiated rectum samples. We observed a significant increase in the content of connective tissue structures and a decrease in the volume of smooth muscle structures, a weak chaotic course of fibers, collagen fiber breaks, and lack of homogeneity. These changes were not revealed in the histological study.

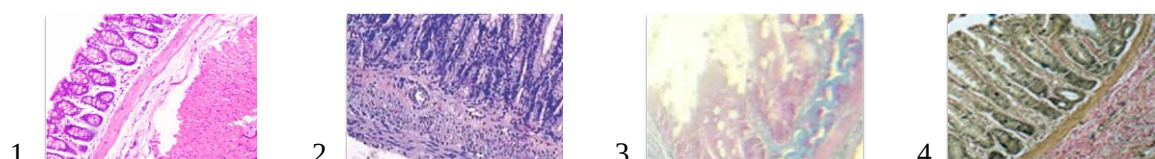


Fig. 3. Histological images of rat rectum samples: 1 – intact; 2 – 10 Gy, one week (hematoxylin and eosin);
3 – 10 Gy, one week (toluidine blue); 4 – 10 Gy, one week (Van Gieson)

The general architectonics of bladder and rectum, according to the PS-OCT data, was not altered independent of the dose and time after irradiation.

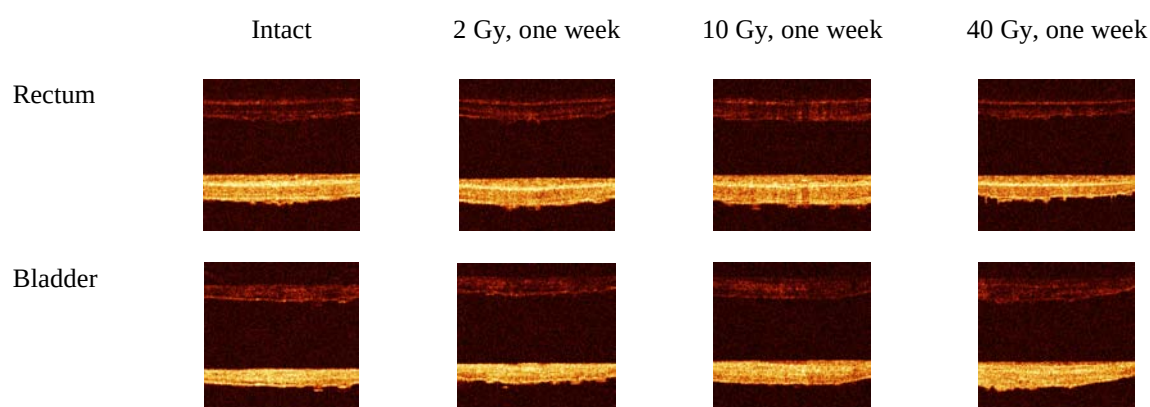


Fig. 4. General architectonics of bladder and rectum

Conclusions

Alteration of extracellular matrix of connective tissue elements of rectal and bladder mucosa and submucosa were observed after irradiation with doses of 2 to 40 Gy.

Acknowledgements

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MULTIPHOTON/CARS TOMOGRAPHY FOR SMALL ANIMAL RESEARCH AND CLINICAL STUDIES

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Abstract. The novel certified clinical multiphoton tomograph MPTflex-CARS was launched in May 2013 during the World-of-Photonics conference LASER2013. The tomograph with its flexible scan head is based on femtosecond near infrared laser pulses for *in vivo* label-free high-resolution tissue imaging due to autofluorescence, second harmonic generation, and CARS. Optical biopsies with subcellular resolution, picosecond temporal resolution, deep-tissue information, and chemical fingerprints can be generated. Wide-field imaging tools such as dermoscopes and optical coherence tomographs can be integrated.

Multiphoton tomographs have been used to detect fluorescent proteins in transgenic mice for stem cell and cancer research as well as non-invasive clinical imaging tools for thousands of patients and volunteers in Australia, Japan, California, and Europe. Skin cancer such as *malignant melanoma* as well as *dermatitis* can be detected at an early stage and the efficiency of the treatment can be monitored. Furthermore, it has been used to detect cosmetics and pharmaceutical components such as sunscreen nanoparticles and anti-aging products in humans. These novel multimodal multiphoton tomographs have been recently employed in space medicine and brain tumor surgery.

Acknowledgements

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A CONFOCAL MICROSCOPY STUDY OF SURFACE BOUND PHOSPHATASE ACTIVITY IN ECTOMYCORRHIZAL FUNGUS *SCLERODERMA* SP. GROWING UNDER DIFFERENT TEMPERATURE CONDITIONS

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Abstract. The influence of different growth temperatures on surface bound phosphatase activity of mycelium of the ectomycorrhizal fungus *Scleroderma* sp. growing in symbiosis with European beech (*Fagussylvatica*) was studied with two photon confocal laser scanning microscopy (CLSM) using the fluorogenic substrate ELF-97. The applicability of the CLSM and image analyses approaches is discussed.

Introduction

Ectomycorrhiza is a symbiotic organ formed by plant fine roots and a fungus, which helps the plant absorb water and nutrients and in which the fungus obtains carbohydrates from the plant. Ectomycorrhizal fungi (EMF) can help plants in utilizing phosphorus (P), protect them from root pathogens and provide resistance to drought [1]. Different factors, biotic and abiotic, can impact surface bound phosphatase activity (SBPA) [2-4].

In our study, confocal microscopy (CLSM) and image analysis were used to study the impact of different temperature conditions on SBPA of ectomycorrhizal fungi (*Scleroderma* sp.) of beech seedlings (*Fagussylvatica* L.) grown in rhizotrons, and the method is discussed regarding the advantages or disadvantages of each step of the methods applied.

Plant and fungal material

Seedlings were grown for three years in glass rhizotrons (30x50x2 cm) at outside air temperatures (LO), in a glasshouse where summer temperature reached over 30°C (RA) and in a temperature controlled walk in growth chamber at 15-20°C without additional cooling of root systems (NHL) and with an additional cooling of the root systems for 4°C (DHL).

The confocal microscopy method and image analysis

Occurrence of surface bound phosphatase (SBP) of *Scleroderma* sp. was investigated with image processed two photon confocal laser scanning microscopy (LSM 7 MP, Zeiss) using the fluorogenic substrate ELF-97 (enzyme-labelled fluorescence; [5]) and computer analyses of images, based on the programme Image J 1.35j.

Staining using **ELF@97 phosphatase substrate (E-6588)** was done on washed mycorrhizal root tips in Eppendorf tubes, which were incubated at room temperature for 15 minutes after the addition of 44 µl of ELF@97 substrate solution (pH 5), followed by rinsing with 100 µl of citrate-phosphate buffer solution (pH 5) to stop enzymatic reaction.

Image analyses with programme Image J1.46r was based on exported files using the programme ZEN[®] 2011, Release Version 7.0, with separated image on channels, where only green was left active. Using POLYGON, the entire root surface (mycorrhizal mantle), hyphae or rhizomorf, to be analysed was selected, based on analysis of the characteristics AREA and AVERAGE FLOURESCENCE INTENSITY. It was tested on original images and in combination of duplicated images.

Results and discussion

The CLSM method

Advantages: i) Vital staining allows visualization of the metabolically active fungal tissue; ii) No clearing of roots is needed prior to staining; iii) Using two-photon confocal microscope does not imply any cross-section of root tips; iv) It could be applied with fresh extracted samples from the experimental area (no pure cultures with cultivated seedlings are needed); v) It is a fast and simple method.

Disadvantages: i) It is a destructive method; ii) Different factors can influence SBP activity, such as pH, temperature, P and N composition in soil, age of mycorrhizae, plant symbiont species or physiology.

Image analyses with the programme Image J1.46r of original images

In the original image analysis the area occupied by the sample is measured. The results are average intensity of the area for each sample.

Disadvantages: the analyses of intensity on original images are suitable if all conditions between parallel images are the same (the same staining, the same power of laser, photomultiplier...).

The solution: more repeatable and also more simple is to analyze contrasting images, therefore measuring the area above threshold. It is possible to choose the threshold, which most effectively cuts off all non-specific staining.

Image analyses with the programme Image J1.46r of duplicated images

Advantages: i) It is easy to select the measuring area; ii) High accuracy of the selection; iii) It is a simple and transparent programme for use.

Dissadvantages: i) Caution is needed in the work order (when the UNDO function is used the measurements can be wrong); ii) If more images are opened (original and duplicates), it is important that for each step (function) a separate click on image is done; iii) It is not possible to export data (measurements).

Why not using any other programme, such as Adobe® Photoshop® CS3 Extended, version 10.0?

Because it is difficult to select the measuring area (the work space area) and the accuracy of the selection is low (when using MagicWandTool).

SBPA activity in differently grown plant-fungus conditions

At the threshold over 10% intensity, the average area of SBPA in NHL was 19% of measuring area, in DHL 18%, in RA 16% and at LO it was 12%.

Therefore we could not statistically prove the differences between treatments, but trends could be recognized, and additional measurements will be done to statistically prove the results.

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STUDY OF CONTRASTING PROPERTIES OF NANOPARTICLES FOR DIFFUSE OPTICAL SPECTROSCOPY APPLICATIONS

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Abstract. We report the results of studying gold and silica nanoparticles as contrasting agents for diffuse optical spectroscopy (DOS). Optical properties of nanoparticles suspensions and model objects were obtained from spectrophotometric measurements in the wavelength range of 500-1100 nm. The DOS measurements were performed with a liquid phantom consisting of Lipofundin and ink in a proportion ensuring optical properties close to that of biological tissue. The phantom contained an inclusion filled with the selected suspension of nanoparticles. The inclusion contrast in the obtained DOS images has been estimated, which allows considering the selected nanoparticles as potentially perspective contrast agents for DOS applications.

The diffuse optical spectroscopy (DOS) is widely used in clinical and biomedical studies [1]. However, this method has a significant disadvantage: low resolution and contrast of the obtained images. One possibility to increase the contrast is to use exogenous contrast agents, such as colloidal gold nanoparticles [2], nanorods, etc. to control the optical properties of biological tissues. The potential of these agents is related to their low cytotoxicity and maintenance of localized surface plasmon resonance in the near-infrared range, which leads to greater reflection and/or absorption of optical radiation by these nanoobjects.

In this study several types of nanoparticle suspensions in water solution were considered: suspension of arbitrary shaped gold nanoparticles (GNPs) with typical size of 30-100 nm (type 1); suspension of arbitrary shaped GNPs with small fraction of nanorods with a length of 30-50 nm and length-to-width aspect ratio 3 (type 2); suspension of Si nanoparticles with sizes of 50-200 nm (type 3).

In DOS experiments we used liquid phantom of 1.4 % solution of lipofundin in water (extinction coefficient $\mu_t = 14 \text{ cm}^{-1}$ for $\lambda = 802 \text{ nm}$) in a plastic container as a model medium mimicking soft tissues of mice [3]. As an inclusion modeling a tumor labeled with contrasting nanoagents we used a microtube filled with a suspension of nanoparticles and a solution of lipofundin with a concentration corresponding to that of the surrounding liquid phantom.

Scattering and absorption coefficient spectra of gold and silicon nanoparticles suspensions were reconstructed from the data of spectrophotometric measurements in the wavelength range of 500-1100 nm using the modified Lambert's law (Fig. 1). Absorption coefficient of all nanoparticles significantly exceeds the scattering coefficient, which makes them potential contrasting agents for DOS. Spectra of GNP (type 1, 2) are characterized by peaks typical for plasmon resonance particles. For type 2 particles the resonances are manifested at 580 nm, which is associated with the plasma frequency of gold, refractive index of the surrounding medium and the size of the particles, and 885 nm which is due to the presence of the fraction of nanorods with length-to-width aspect ratio of about 3. For type 1 particles the first resonance is at the wavelength of 645 nm, while the second one related to the particles shape is possibly shifted to the IR region.

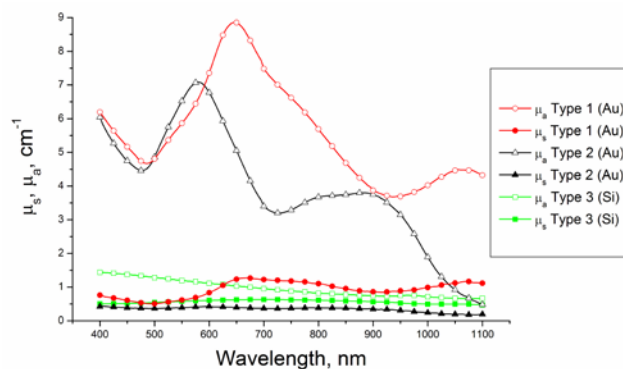


Fig. 1. Optical properties of gold and silicon nanoparticles suspensions

To study the effect of nanoparticles on the contrast of diffuse tomography images we employed the DOS experimental setup developed at the Institute of Applied Physics RAS. The system is equipped with three semiconductor laser sources with wavelengths of 657, 802, and 919 nm. For experiments we chose nanoparticles of type 1 and 2 demonstrating quite stronger absorption at all three operating wavelengths of the DOS system compared to suspension of Si nanoparticles. Contrast of the inclusion in the obtained DOS images was defined as $C = (I_2 - I_1)/(I_2 + I_1)$, where I_1 is the value of the registered signal in the region corresponding to the microtube, while I_2 corresponds to the average signal from the region without the tube. Preliminary model experiments with ink-filled microtube were conducted to determine the sensitivity of the DOS-image contrast to the presence of absorbing inclusion. Dependence of the contrast on the volume concentration of ink is shown in Fig. 3 (a).

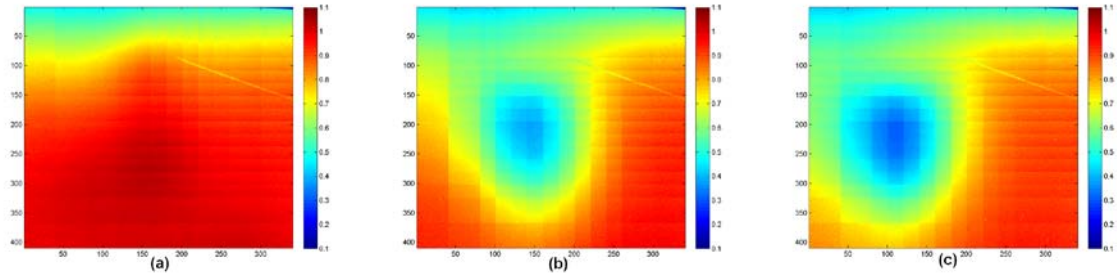


Fig. 2. DOS-images ($\lambda = 802$ nm) of lipofundin (1.4%) phantom with a tube filled with (a) lipofundin, (b) lipofundin + suspension of GNPs (type 1), (c) lipofundin + suspension of GNPs (type 2). Corresponding extinction coefficients within tube: 14 (a), 18.2 (b) and 20.9 (c) cm^{-1}

At the next step the experiments on the DOS images contrasting by GNPs were conducted. The obtained images are shown in Fig. 2. The corresponding quantitative evaluation of the tube contrast in DOS images for gold nanoparticles as a function of wavelength is shown in Fig. 3(b). The obtained values of contrast are in good agreement with the results of model experiments with ink, which shows a promising potential of the selected suspensions of gold nanoparticles as contrast agents for DOS applications. However, the behavior of the contrast dependence on the wavelength does not correspond to the absorbing ability of the embedded inclusion which is mostly determined by the absorption spectrum of GNP suspension. We suppose that this effect is caused by aggregation of the nanoparticles in lipofundin solution and by optical properties of water.

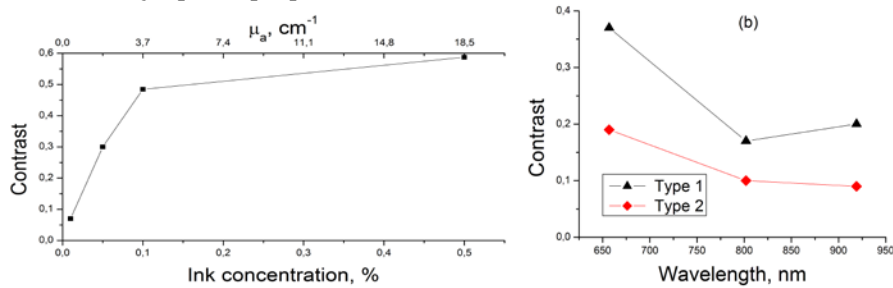


Fig. 3. Dependence of the microtube contrast in DOS images on a) volume concentration of ink in the tube ($\lambda = 802$ nm); b) wavelength (obtained in the experiment with suspensions of GNP (type 1, 2))

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OPTICAL COHERENCE ELASTOGRAPHY: A BRIEF REVIEW OF CURRENT TECHNIQUES AND CHALLENGES

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Abstract. Mechanical forces play an important role in the behavior and development at all spatial scales, from cells and their constituents, to tissues and organs. Such forces have a profound influence on the health, structural integrity, and normal function of cells and organs. Accurate determination of tissue biomechanical properties (e.g., Young's or shear modulus) could help with clinical diagnosis and interpretation of various diseases. In this talk I'll overview recent advances on developments and application of optical methods to assess mechanical properties of different tissues and show examples of the current state-of-the-art optical elastography imaging of tissues, including tumors and ocular tissues.

Elasticity imaging (elastography) is a technique capable of remote mapping of the mechanical properties of tissue. Elastography relies on applying a small stimulus to the tissue, and analyzing the tissue mechanical response. A number of imaging techniques have been used for elastography, including ultrasound imaging, Magnetic Resonance (MRI), Atomic Force Microscopy (AFM) imaging, and optical and microscopic imaging techniques. In comparison with other imaging techniques, in particular high frequency ultrasound imaging, OCT has advantages in resolution and, specifically for ophthalmological applications, penetration depth [1, 2]. OCT can image nanometer level tissue displacements induced by compression forces [3-9].

OCE techniques generally rely on a loading system which generates dynamic or static excitation of tissue deformation and apply an OCT-based sensing method to capture and measure the tissue displacement with high sensitivity [10]. The tissue excitation methods can also be classified as internal or external excitation based on their spatial characteristics. For the detection of tissue deformation, conventional methods mainly employed speckle-tracking algorithms [11, 12]. Recently, the rapid development of phase-resolved OCT techniques allows improving the accuracy of the detection reaching nanometer-scale [13, 14]. Current OCE methods probe and provide two types of information concerning the tissue biomechanical properties: one is related to the local displacement or strain inside the tissue under compressive stress; the other is the shear modulus or Young's modulus of tissue obtained from the elastic models with quantification approaches.

Optical coherence elastography (OCE) was introduced by Schmitt in 1998 to measure the microscopic deformation of biological tissue under compressive stress induced by a piezoelectric actuator [8]. Two-dimensional cross-correlation speckle tracking was applied for measuring the internal tissue displacement with micrometer-scale. This work also provided the first demonstration for the *in vivo* application of OCE.

To image and map the displacement-related or strain-related parameters inside tissue, dynamic OCE techniques have been extensively studied. Adie et al. developed an audio-frequency OCE method for which a piezoelectric transducer was used to excite tissue deformation and an OCT system applying balanced optical quadrature detection was utilized to monitor the spatial distribution of the tissue displacement [15]. With a fixed driving frequency, the dynamic differential strain was quantified and related to the localized elastic properties of a layered phantom and *in vivo* human skin. Also employing a piezoelectric transducer for dynamic external excitation, Liang et al. utilized a phase-sensitive OCT system to assess the tissue displacement and quantified the localized strain rate inside the tissue [6]. The experimental results with a layered phantom indicated that the local strain rate increases with the vibration frequency getting close to the resonance frequency of a particular layer. By applying different driving frequencies, the feasibility of this method in characterizing *ex vivo* rat tumor tissue has been demonstrated with two-dimensional mapping of the local strain rate. This method was then further developed by Kennedy et al. with the dynamic excitation from a ring actuator operated at a single frequency [16]. With this OCE system, three-dimensional imaging of the strain rate for *in vivo* human skin with high spatial resolution was demonstrated for the first time.

Besides utilizing the strain-related information, Adie et al developed a spectroscopic OCE method where the frequency-dependent complex mechanical response including the amplitude and the phase of the localized displacement from tissue were extracted and utilized for the contrast of tissue elastography [17]. The external piezoelectric transducer was operated at audio-frequency range for excita-

tion and phase-resolved OCT detection was applied. The feasibility of this method has been demonstrated for *ex vivo* heterogeneous samples, including rat tumor and muscle. In addition, with the acoustic radiation force provided by a piezoelectric transducer as the external excitation, Qi et al. utilized a phase-sensitive OCT system to measure the local displacement inside tissue, and reconstructed a three-dimensional map of the tissue vibration amplitude for the elastography of *ex vivo* artery tissue with atherosclerotic lesion [18]. Instead of using external loading system, Liang et al. developed an internal dynamic excitation method with the oscillation of magnetic nanoparticles inside the tissue remotely controlled by a magnetic field [19]. Recently, Kennedy et al. investigated the feasibility of integrating OCE into a needle [20]. This needle OCE system measured the microscopic displacement deep inside the tissue as the representation of the tissue mechanical properties. The force imparted by the needle tip was applied as the loading mechanism for the localized tissue excitation. Experiments performed on *ex vivo* porcine trachea demonstrated the feasibility of this needle-based method in depth-resolved elastography.

Most recently, the group of R. Wang [21] and our group [22-25] have demonstrated capability of phase-sensitive OCT to assess nm-amplitude mechanical waves propagating on tissue surface. We also just demonstrated, for the first time (to the best of our knowledge), a possibility of quantification of shear wave parameters in live mice cornea *in vivo* and with very low (sub μm) excitation using PhS-SSOCT technique [22]. In this talk I'll overview recent advances on developments and application of OCE to assess mechanical properties of different tissues.

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NUMERICAL ABERRATION CORRECTION METHOD FOR DIGITAL HOLOGRAPHY OPTICAL COHERENT TOMOGRAPHY

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One of the most popular methods of correction of optical aberration consists in the use of reference phase front. As a source for reference phase front one can use a specially induced pinpoint scatterer or flat region of target. Unfortunately, the use of this method is significantly complicated for some biological objects such as human eye. Another method is based on mutual matching of a number of parameters for maximizing certain metric.

The work concerns development of a numerical aberration correction method based on the use of spatially selective filters for Fourier image of phase distortion distribution caused by optical aberrations.

The proposed method was verified experimentally using a Fourier-spaced digital holography setup in a reference wave tilted mode.

CORRELATION-STABILITY APPROACH IN OCT ELASTOGRAPHY: POSSIBILITIES OF USING DIFFERENT-SCALE FEATURES OF OCT IMAGES

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Abstract. Inhomogeneity of the OCT image intensity is determined by two essentially different factors: (1) inhomogeneity of light scatterer distribution on a scale exceeding the resolution of the OCT scanner (morphological structure); (2) the presence of a few sub-resolution scatterers within one resolution volume, which leads to appearance of a speckle structure. If the tissue is deformed, both speckle-related and morphology-related image structures are distorted, so that the cross-correlation between the consequent tissue images is reduced. The degree of this distortion is less in regions with increased stiffness. Instead of the frequently discussed direct estimation of displacements and then reconstruction of local strains, the degree of correlation stability (CS) can be used for characterizing relative stiffness of tissue.

Introduction

Conventional OCT images characterize the inhomogeneity of scattered-light intensity in a single frame with a typical size of a few hundred by a few hundred pixels. This pixelized structure is related to system resolution determined by the size of light beams in lateral direction and by the coherence length of the light source in axial direction. The intensity inhomogeneity of the resulting image is determined by two essentially different factors. One of them is the inhomogeneity of light scatterer distribution on the scale exceeding the image element resolution (ranging from a few pixels to the frame size) and related to the morphological structure of the biological tissues. The other factor concerns the presence of several subresolution scatterers in the resolution volume. Interference of the contributions of the subresolution scatterers leads to formation of a specific speckle structure of OCT images superposed with larger-scale inhomogeneity determined by morphological structure. If the tissue is deformed, both speckle-related and morphology-related image inhomogeneities are distorted. The distortion comprises both translational motion of image fragments and deformation of their shape. The degree of the latter is less in regions with increased stiffness. Instead of the frequently discussed [1-3] direct estimation of displacements (using sliding correlation windows) and then reconstruction of local strains (which is a rather error-sensitive procedure), the degree of correlation stability (CS) can be used for characterizing relative stiffness of the tissue [4,5]. Basically, the procedure of cross-correlation between subsets (usually rectangular windows of $m_1 \times m_2$ elements) from the reference and deformed images is similar to that used, e.g., in [1-5]:

$$C(n, k) = \frac{\sum_{i=1}^{m_1} \sum_{j=1}^{m_2} (S_{i,j} - \mu_S)(F_{i+n, j+k} - \mu_F)}{\left[\sum_{i=1}^{m_1} \sum_{j=1}^{m_2} (S_{i,j} - \mu_S)^2 \cdot \sum_{i=1}^{m_1} \sum_{j=1}^{m_2} (F_{i+n, j+k} - \mu_F)^2 \right]^{1/2}}, \quad (1)$$

where the subset window S of $m_1 \times m_2$ elements from the reference image is moved over the $n \times k$ pixels search area and is correlated with similar in size areas F of the deformed image (as is described, for example, in [4,5]), μ_S and μ_F are the respective mean values over the correlated areas in the reference and deformed images. For identical areas, the coefficient $C=1$ and tends to zero for completely uncorrelated areas. Typical values for $m_{1,2}$ are 10-40 pixels [1-5].

The CS-approach to purely morphological distortions

First, we consider the case of OCT images with morphological structure [4, 5] that is realized if the speckles in OCT images can be filtered out or the scatterer distribution in the tissue does not create speckles. We demonstrate applicability of the CS-approach for this case in Figs. 1 and 2. Figure 1 (a) is the simulation of an OCT-image with a stiffer thin layer (invisible in the OCT-image) in the middle of the sample, Fig. 1 (b) is the laterally deformed image from Fig. 1 (a), and Fig. 1 (c) is the elastographic image

based on the CS-approach. Elastographic images obtained *in vivo* using the CS-approach are shown in Fig. 2. For *in vivo* examples we have chosen human cheek skin where hair roots serve as stiffer inclusions in Fig. 2 (a, b), and Fig. 2 (c) shows a stiffer inclusion revealed in the mucous part of human lip.

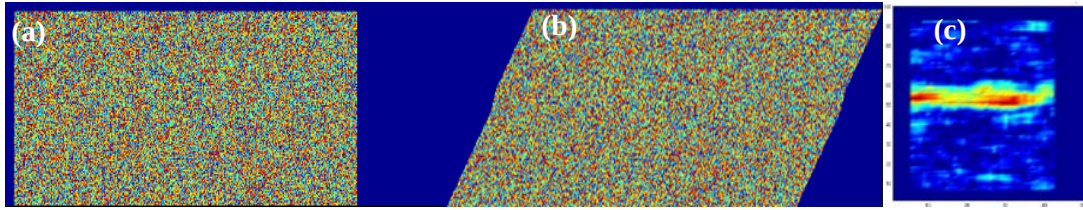


Fig. 1. Panel (a) simulated OCT image with stiffer layer in the middle, panel (b) laterally deformed OCT image, panel (c) elastography map based on CS-approach

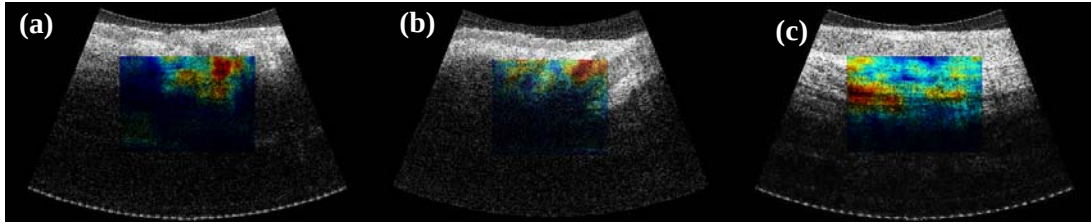


Fig. 2. Elastography map based on CS-approach for human cheek skin where hair roots serve as stiff inclusions – panels (a, b) and stiffer inclusion in the mucous part of human lip – panel (c)

The Cs-approach to the speckle-formed OCT-images

The most important case is the OCT-image fully formed by speckles. Speckle decorrelation during deformation process usually occurs earlier than the decorrelation induced by purely geometrical distortion. In the case of speckles, the CS-approach is applicable too, but it requires sufficiently high speed of OCT to avoid complete decorrelation of consequent scans. In this part of the presentation we demonstrate the applicability of the CS-approach to the case of speckles. Figure 3 (a) presents a simulated two-layer OCT image formed purely by speckles, Fig. 3 (b) is the elastographic map based on the CS-approach, Fig. 3 (c) shows a real OCT image of a two-layer phantom, and Fig. 3 (d) is the corresponding elastography map based on the CS-approach.

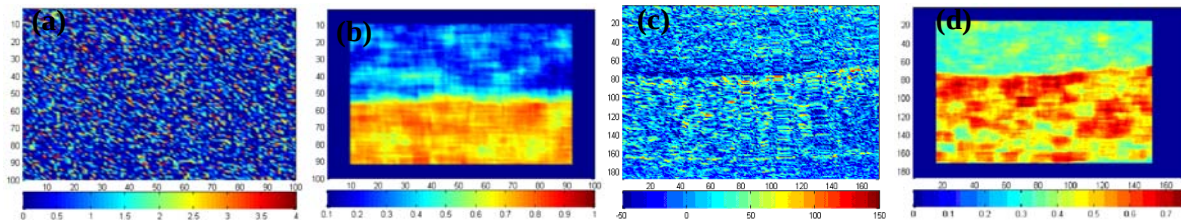


Fig. 3. Panel (a) is the simulated two-layer OCT image, panel (b) is the corresponding elastography map based on the CS-approach, panel (c) is the OCT image of two layer phantom (with ~ 3 times stiffness contrast), and panel (d) is the corresponding elastographic map based on CS-approach

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STUDY OF THE ACTION OF LOCAL PHOTODYNAMIC THERAPY ON THE GROWTH OF PRIMARY TUMOR AND DEVELOPMENT OF METASTASES

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Abstract. The goals of our work were to study the action of PDT and PDT combined with the use of immunoadjuvant on the growth of primary tumor and development of metastases of mice melanoma. Stable fluorescent cell line B16-mKate2 was established. Photosensitizers show effective phototoxic action *in vitro* on B16 and B16-mKate2 cells. Significant inhibition of tumor growth and reduction of the number of lung metastases after PDT of B16-mKate2 tumors with bacteriopurpurinimide derivative was demonstrated. Subcutaneous application of complete Freund immunoadjuvant combined with PDT of lung metastases under certain conditions causes decreasing of lung metastases.

Photodynamic therapy (PDT) is based on the use of natural and synthetic photosensitizers (PS) that selectively accumulate in tumor cells and cause their death when irradiated with light of a specific wavelength. At present, researchers consider several key components of the mechanism of antitumor action of PDT: direct damage of tumor cells, damage of tumor vascular system, stimulation of the immune response, etc. [1, 2].

Chen and co-authors [3] consider that the combination of PDT and immunoadjuvant enhances the antitumor effect on primary tumors in rats and mice and inhibits the growth of metastases in the lymph nodes. However, the impact of PDT and PDT in combination with immunoadjuvants on hematogenous metastasis has not been studied until now.

The goals of our work were to study of action of PDT and PDT combined with using immunoadjuvant on the growth of primary tumor and development of metastases of mice melanoma.

Materials and Methods

The following cell lines were used for our experiments both *in vitro* and *in vivo*: mice melanoma B16 and fluorescent cell line based on B16. A stable fluorescent cell line expressing far-red fluorescent protein mKate2 (fluorescence with excitation/emission maxima at 588 and 633 nm) was established by lipofection of B16 cells using plasmid pmKate2-N (“Evrogen”, Russia) with subsequent selection by geneticine and cloning.

To obtain xenograft tumor model mice melanoma cell lines B16 and B16-mKate2 were used. Cells (10^6 in 50 μ L) were inoculated subcutaneously into the back of C57Bl/6 mice (breeding of Russian Oncologic Scientific Center).

For studying phototoxic action *in vitro* and photodynamic action *in vivo* the following photosensitizers were used: methyl ether of O-ethyloxime-N-ethoxybacteriopurpurinimide (M.V. Lomonosov Moscow State University of Fine Chemical Technologies, Russia) and bacteriochlorine tosylate (State Scientific Center “NIOPIC”) with irradiation of cells and tumors by lasers Biospec LFT-800-01 at 800 nm (for the first PS) and Biospec LIMIS (“Biospec, Russia”) at 775 nm (for the second PS).

Tumor growth and inhibition was estimated by palpation as well by different fluorescence methods, such as fluorescent imaging by fluorescence small animal imaging system UVP iBox (UVP, LLS, USA).

Results and Discussion

A stable fluorescent cell line expressing far-red fluorescent protein was established. There were no significant differences in tumor growth between B16 cell line and fluorescent cell line B16-mKate2 at subcutaneous inoculation of cell suspension.

It was shown that both photosensitizers show effective phototoxic action *in vitro* on B16 and fluorescent B16 cells used at non-cytotoxic concentrations.

Monitoring of tumor growth of B16-mKate2 tumors after PDT (dose of methyl ether of O-ethyloxime-N-ethoxybacteriopurpurinimide – 2 mg/kg of bodyweight, irradiation 2.5 hours after administration of PS at a power density of 150 mW/cm² for 15 minutes) showed that the inhibition of

tumor growth was maintained at 84 % for 2 weeks after PDT. The PDT of primary tumor with this PS significantly reduced the number of distant (particularly lung) metastases.

It was shown that PDT on lung metastases model using bacteriochlorine tosylate (1 mg/kg of bodyweight i.v. 30 min before PDT, 36 mWt/cm²) didn't affect lung methastases 2 weeks after PDT, there were no significant differences between metastases in the treated and the control groups.

It was found that subcutaneous use of complete Freund immunoadjuvant combined with PDT on lung metastases model using methyl ether of O-ethyloxime-N-ethoxybacteriopurpurinimide under certain conditions causes a decrease of lung methastases 2 weeks after PDT compared with the control.

Tumor cells isolated from tumor not subjected to PDT effectively formed on Matrigel Matrix (BD Biosciences, USA) vasculogenic-like network, unlike tumor cells undergoing PDT both with and without immunoadjuvant.

Acknowledgements

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METHOD OF PERFORMING NONUNIFORM FOURIER TRANSFORM AND ITS VALIDATION BY SPECTRAL DOMAIN OPTICAL COHERENCE TOMOGRAPHY DATA

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Abstract. In this paper we introduce a method of performing Fourier transformation of nonequidistantly spaced data. We show that data resampling can be performed as “nonuniform convolution”, i.e. convolution with the kernel the shape of which depends on position. Because the length of such a kernel can be chosen to be relatively small (about 9 elements) and no data upsampling is required, this operation can be efficiently computed numerically. The method was validated by Spectral Domain Optical Coherence Tomography (SD OCT) data.

In different practical problems from radio astronomy to medical imaging, such as magnetic resonance imaging and spectral domain optical coherence tomography (SD OCT), frequency data is used to generate images. If data is sampled on a Cartesian grid, images can be computed by the Fast Fourier Transform (FFT). This condition is not always met in real experimental data. In SD OCT, for instance, data is acquired on the Cartesian grid in λ , while for correct imaging data sampled equidistantly in k is required. Since direct computation of nonuniform Fourier Transform (NUFT) requires $O(N^2)$ operations, different algorithms can be applied to solve this problem, such as different types of data resampling [1-3], gridding [4, 5], and Lomb periodogram [6]. Here we will describe one more method of performing NUFT and will show its efficiency on SD OCT data.

Consider the case of transformation from non Cartesian “k-space” to Cartesian “z-space”. Straightforward computation of the NUFT of the nonequidistantly sampled vector g_k is matrix to vector multiplication:

$$\tilde{g}_n = \hat{M} \times g_k, \quad (1)$$

where elements of the matrix $\hat{M}$ are $\hat{M}_{n,k} = \exp(-i \cdot 2 \cdot \pi \cdot n \cdot (k + \Delta k(k)) / N)$, where $\Delta k(k)$ represents the difference in number k from the node of the Cartesian grid. We can preserve the equality (1) by multiplying the right-hand side of the equation on identity matrix:

$$\tilde{g}_n = \hat{M} \times g_k = \hat{I} \times \hat{M} \times g_k = (\mathbf{FT} \times \mathbf{iFT}) \times \hat{M} \times g_k = \mathbf{FT} \times (\mathbf{iFT} \times \hat{M}) \times g_k = \mathbf{FT} \times \hat{A} \times g_k. \quad (2)$$

Here $\mathbf{FT}$ and $\mathbf{iFT}$ represent matrices of Discrete Fourier transform and its inverse. Multiplication of matrix $\hat{A}$ to vector g_k resamples g_k onto Cartesian grid. Let’s take a look on the matrix $\hat{A}$. We can note that this matrix is sparse: amplitudes of some of its elements are much higher than of the other. The basic idea of the method proposed in the work is to preserve only m biggest elements in each row of $\hat{A}$ and replace matrix to vector multiplication by “nonuniform convolution”. As the phase of the coefficients of the NUFT matrix $\hat{M}$ grows linearly along columns, the coefficients of the transformed matrix $\hat{A}$ can be easily calculated:

$$\begin{aligned} \tilde{g}_n &= \hat{A} \times g_k \approx \sum_{k_{\min}(n)}^{k_{\max}(n)} C_{n,k} \cdot g_k, \\ C_{n,k} &= \frac{\exp[i \cdot 2 \cdot \pi \cdot (k + \Delta k(k) - n)] - 1}{i \cdot \frac{2 \cdot \pi}{N} \cdot (k + \Delta k(k) - n)}. \end{aligned} \quad (3)$$

Here both first and last numbers in the summation depend on the number of the calculated element. The difference $k_{\max} - k_{\min}$ is always equal to m – the number of remaining elements in the row of $\hat{A}$. $C_{n,k}$ can be precalculated and stored as well for each dependence $\Delta k(k)$. Thus, now NUFT requires $O(m \times N)$ plus $O(N \log N)$ operations needed for FFT.

Direct implementation of the described method may cause some blurring at low depths. To overcome this drawback, two possible ways were proposed.

The first one can shift low frequencies to the region of higher frequencies, multiplying the initial spectrum by the appropriate exponent according to the shift theorem of the FT. The result should be shifted back. In this case we can write the coefficients $C_{n,k}$ as:

$$C_{n,k} = \frac{\exp\left[i \cdot 2 \cdot \pi \cdot (k + \Delta k(k) - n)\right] - 1}{i \cdot \frac{2 \cdot \pi}{N} \cdot (k + \Delta k(k) - n)} \cdot \exp\left[i \cdot 2 \cdot \pi \cdot S \cdot (k + \Delta k(k)) / N\right] \cdot \exp\left[-i \cdot 2 \cdot \pi \cdot S \cdot n / N\right], \quad (4)$$

where S is the size of the shift. Low negative frequencies are obtained in the same way by shifting them to the left and back again. The resultant “z-space” distribution is obtained by fusion of two “z-space” distributions obtained by shifting the initial “z-space” distribution to different directions.

However, in many arrangements of the SD OCT setup only half of z-space distribution is used, while the second half represents a mirror image. Even if not so, we always can expect that part of the OCT image carries no information and represents only multiple scattering. Thus, we can use the shift tactic and make shift only in one direction without any fusion. This allows obtaining SD OCT images only in one “nonuniform convolution” with further FFT. The results of applying different techniques to the OCT data are shown in Fig. 1. (We choose a 9-elements long kernel because it doubles the number of calculations in the case of 512 samples long realization, while satisfactory results may be obtained with a 5-elements long kernel).

Another way of improvement is to pad columns of matrix $\hat{M}$ with zeros, i.e. construct a matrix, each column of which will be organized, for example, as follows: $N/2$ zeros, N elements of matrix $\hat{M}$, $N/2$ zeros, inverse Fourier transform to this matrix to obtain coefficients which we should also place in the first equation in system 3 (n now changes from 0 to $2N$). (If we place zeros in another place (e.g. N zeros followed by N elements of matrix $\hat{M}$), we only should appropriately change the corresponding coefficients). After FFT of the product of the padded matrix by the spectrum, we should take as a result part of the “z-space” distribution with indexes from $N/2$ to $N/2+N$ in the present case.

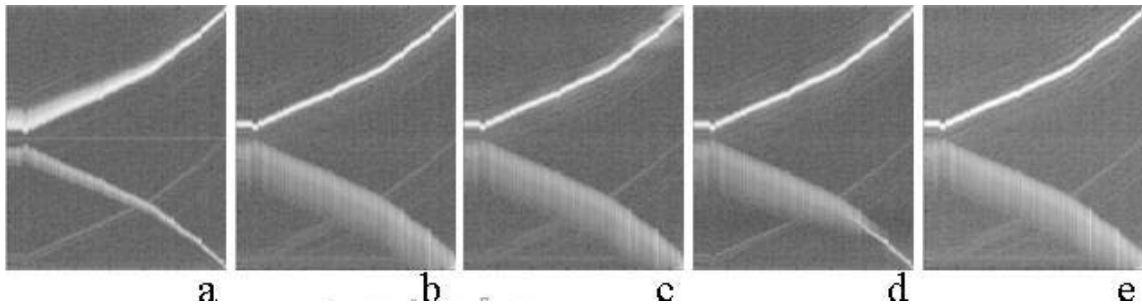


Fig. 1. Image of the surface obtained with SD OCT setup after direct FFT (a), NUFT matrix to vector multiplication (b) “nonuniform convolution” (9 elements kernel). One can see blurring of low frequencies (c), “nonuniform convolution with shift” (9 elements kernel). One can see that part of the mirror image also became sharper (d), “nonuniform convolution with padding” (9 elements kernel) (e)

Note, that kernel of the same size shows the same performance with different samples lengths. Thus, for SD OCT data with 512 samples the proposed “shift technique” requires only 2 times more computations than direct FFT.

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INTERFEROMETRIC SYNTHETIC APERTURE MICROSCOPY WITH AUTOMATED PARAMETER EVALUATION AND PHASE EQUALIZATION

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Abstract. A method of OCT imaging with a resolution throughout the investigated volume equal to the resolution in the best-focused region is described. A method of finding parameters needed for algorithmic realization of the summation is also proposed.

In Spectral Domain OCT (SD OCT) axial distribution of the field scattered by the object is registered (A-scan) by measuring several spectra of interference between the required and reference fields with different reciprocal phase shifts. In OCT imaging, several consecutive A-scans form the image of a near-surface layer of the studied object (B-scan) [1], and from a series of B-scans one can construct 3D distribution of the scattered field [2]. Axial resolution in OCT is defined by the spectral band of the utilized radiation source $\Delta\lambda$. Lateral resolution defined by the focal spot size of the scanning beam is limited by the requirement of uniform in-depth irradiation of the object under study, which is attained by reducing the numerical aperture (NA). In our previous work [3], based on the analogy between broadband digital holography and OCT, we proposed a method of increasing lateral resolution in the whole investigated volume by using high NA (from 0.1 to 0.2) of a scanning beam and a subsequent procedure of resolution enhancement in out-of-focus region by multiple numerical shifts of the focal region. 2D holograms were formed in the X-Y plane on the basis of consecutive pointwise interference spectra measurements for every narrowband spectral component with spectral width $\Delta\lambda/N$ specified by one of N components of spectral decomposition. The number N of decomposition elements should be sufficiently large for the coherence length of each narrowband component to exceed the depth of the investigated region. This approach enables obtaining 3D distribution of scattered field $f(x, y, z, k_i)$ for every component of spectral decomposition with bandwidth $\Delta\lambda/N$ based on solution of the diffraction problem by the angular propagation method. Both lateral and axial resolution in this case are defined by diffractive properties of the scanning beam.

Summation of three-dimensional field distributions over all wavenumbers gives an OCT image $F(x, y, z)$ whose lateral resolution throughout the studied volume is defined by the resolution in the plane of optimal focus, whereas axial resolution is improved to the value determined by the radiation source spectra width. In the small-angle approximation $u^2 + v^2 \ll k^2$ fulfilled in OCT we can write:

$$F(x, y, z) = \sum_k iFT_{u,v \rightarrow x,y} \left(FT_{x,y \rightarrow u,v} \left(f(x, y, 0, k) \right) \cdot \exp \left(i \cdot z \cdot k \cdot \left(1 - \frac{u^2 + v^2}{4 \cdot k^2} \right) \right) \right) \quad (1)$$

It should be noted that image reconstruction throughout the investigated volume by eq. (1) is possible only with predefined initial position of optimal focus plane and index of refraction of the studied medium. Calculation of three-dimensional (depending on u, v, k) spectrum of scatterers' spatial distribution throughout the studied depth by eq. (1) with certain values of angular spectrum u, v is a nonuniform Fourier transform (NUFT) that can be written as a multiplication of initial vector $\tilde{f}(u, v, 0, k) = FT_{x,y \rightarrow u,v} (f(x, y, 0, k))$ by matrix M with elements:

$$M_{n,m} = \exp \left(i \cdot \frac{2\pi \cdot m \cdot n}{N} + i \cdot \alpha (n - N_0) \cdot \frac{(\Delta u \cdot p)^2 + (\Delta v \cdot l)^2}{4 \cdot (k_{\min} + \Delta k \cdot m)} \right), \quad (2)$$

where p and l are the transversal indexes of the angular spectrum, $\Delta u, \Delta v$ are the discretization parameters of the angular spectrum ($u = \Delta u \cdot p, v = \Delta v \cdot l$), N is the number of spectral samples, $k_{\min} = 2 \cdot \pi \cdot n_r / \lambda_{\max}$, $\Delta k = (2 \cdot \pi \cdot n_r / \lambda_{\min} - 2 \cdot \pi \cdot n_r / \lambda_{\max}) / N$; $\lambda_{\min}, \lambda_{\max}$ are the minimal and maximal wavelengths in radiation source spectrum, m is the index in the wavenumber space, n is the index in the Fourier-conjugate space, $z = \alpha \cdot (n - N_0)$, N_0 is the plane of optimal focus, and α is the optical interval between the adjacent planes indexed by n in image space. Note that the dependence $z(n)$ remains linear for any index of refraction distribution, since in SD OCT the measured optical spectrum modulation of interference signal is defined by spectral intervals that are determined by optical distances.

This method operates with a full three-dimensional array of OCT data; thus, usage of iterative algorithms of optimal parameter fitting requires a great amount of calculations. For this reason, in [3] calculations by eq. (2) were replaced by numerical shift of the region of optimal focus on the grid of predefined values Δz with subsequent image synthesis from fragments with best resolution. Numerical focus shift can be written in the form

$$F_1(x, y, z) = \sum_k \exp(i \cdot k \cdot z) \cdot iFT_{u,v \rightarrow x,y} \left(FT_{x,y \rightarrow u,v} (f(x, y, 0, k)) \cdot \exp \left(-i \cdot \Delta z \cdot \frac{u^2 + v^2}{4 \cdot k} \right) \right) \quad (3)$$

This operation shifts the region of optimal focus of the image by distance Δz . With the continuous coordinate z in eq. (2) replaced by its fixed value Δz , the variable index n in eq. (3) is replaced by its value N_i :

$$\tilde{M}_{n,m} = \exp \left(i \cdot \frac{2\pi \cdot m \cdot n}{N} + i \cdot \alpha (N_i - N_0) \cdot \frac{(\Delta u \cdot p)^2 + (\Delta v \cdot l)^2}{4 \cdot (k_{\min} + \Delta k \cdot m)} \right) \quad (4)$$

According to (4), instead of NUFT (2), we multiply the 3D OCT spectrum by a phase mask with subsequent conventional discrete Fourier transform. Assume that parameters α and N_0 are specified so that their substitution into eq. (2) for calculating the distribution with improved lateral resolution throughout the studied volume will correspond to the resolution in the plane of optimal focus. Then the plane of optimal focus after calculations according to eqs. (3,4) will be shifted numerically on the plane with index N_i . If, instead of α and N_0 parameters, α_1 and N_1 are specified, then the focal plane will shift to the plane with index N_j related to N_i by $\alpha_1 \cdot (N_j - N_1) = \alpha \cdot (N_i - N_0)$.

For determining parameters of the linear dependence $\alpha \cdot (N_i - N_0)$ one should find the optical distance from the initial plane of optimal focus to arbitrary two of N X-Y planes in the OCT image. Toward this end, let us numerically shift the focal region by two depths and for each resulting image find a plane located at half the optical distance between the initial and the resulting planes of optimal focus. Physically, the width of spatial spectra of amplitude distribution of the scattered field at this depth will remain the same for the initial image and for the image with numerically shifted focal region. Thus, for finding a required plane we plotted the curve for the ratio of spectral widths of amplitude distributions in initial and numerically refocused images as a function of imaging depth. The obtained curve was low-pass filtered to reduce the influence of noise and its intersection with unity was sought for. Coordinates of the intersection correspond to the required position of $N_{j1/2}$ for every preset value of Δz_j . Thus, we can obtain a set of linear equations and find parameters α , N_0 required for calculating distributions with lateral resolution throughout the studied depth corresponding to the resolution in the focal region.

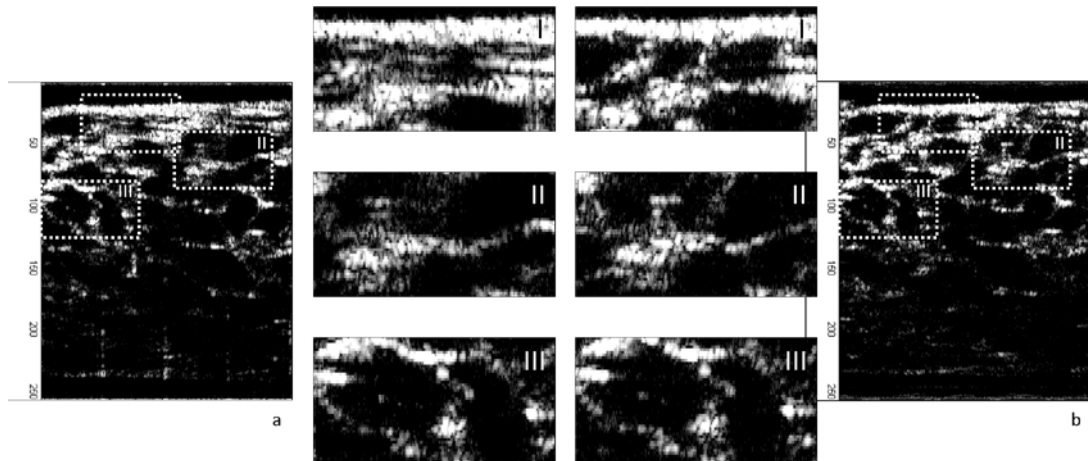


Fig. 1. a – Image of internal structure of an orange pulp acquired with a tightly focused scanning beam. **b** – Image reconstructed according to eq. (2) with parameters determined by images with numerically shifted focal region

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MORPHO-CHEMICAL ANALYSIS OF TISSUES

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Abstract. A review of our approach to morpho-chemical analysis of tissues will be presented. Different modalities related to non-linear and linear measurements will be shown. Also, the combination with non-coherent light for therapy solutions will be demonstrated.

Modern optics and spectroscopy are offering promising non-invasive solutions to potentially improve diagnostic capability on tissues, as demonstrated by the extensive use of non-linear laser scanning microscopy for tissue imaging in the past decade.

The recent development and integration of multiple non-linear microscopy techniques in a single instrument has provided new opportunities for integrating morphological and functional information and for correlating the observed molecular and cellular changes with disease behaviour. In particular, multimodal non-linear imaging is able to perform a morpho-chemical quantitative analysis in tumour cells and tissue specimens, providing a high-resolution label-free alternative to both histological and immunohistochemical examination of tissues.

Although up to now limited to optical research labs, multimodal non-linear imaging is becoming increasingly popular among medical doctors and has the potential to find a stable place in a clinical setting in the near future. Results obtained on various tissues using multiple non-linear imaging techniques are presented.

In the first part of this talk a brief review on the non-linear laser imaging techniques will be displayed. In particular, two photon fluorescence microscopy, lifetime imaging, multispectral imaging, second harmonic generation microscopy principles will be described.

In the second part of the talk there will be an overview on the applications of these techniques in the field of biomedical imaging. In particular, tumor detection in tissue imaging applications will be shown in different fields, from urology to gastrointestinal surgery, dermatology and brain surgery.

Morpho-functional characterization of tissue pathologies will be displayed as an interesting tool for tumor early diagnosis.

In the last part of the talk, a fiber-based endoscope based on multidimensional spectral detection (one photon fluorescence, lifetime and Raman detection) will be described with particular applications of tumor detection.

Finally, a low cost and compact, LED based, imaging solutions, aimed at characterizing the tissues features, will be displayed.

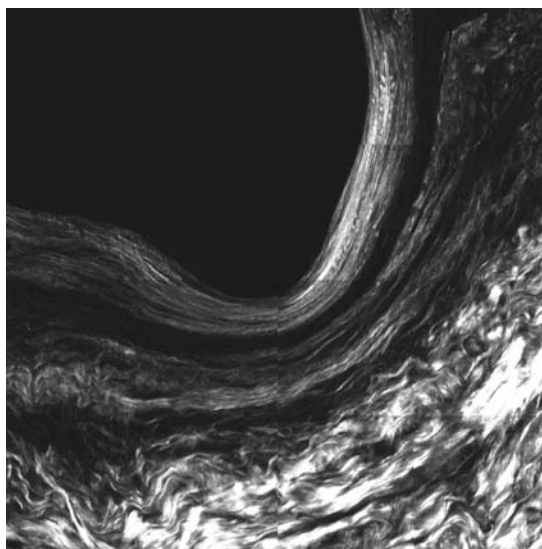


Fig. 1. Collagen fibers (second harmonic signal) and elastin fibers (two photon) in the stroma area of a tumor in a basal cell carcinoma

POINT SPREAD FUNCTIONS OF FOCUSED ULTRASONIC DETECTORS USED IN PHOTOACOUSTIC MICROSCOPY: NUMERICAL CALCULATIONS

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Abstract. Photoacoustic microscopy (PAM) is an effective approach providing 3D photoacoustic imaging without reconstruction. PAM is based on using focused ultrasonic detectors with high numerical aperture (NA), that allow receiving photoacoustic signals from a limited spatial region defined by detector focal spot. However, finiteness of focal spot size leads to blurring of resulting PAM images. Knowing a point spread function (PSF) one can improve PAM images of initial absorption distribution. In this presentation we will demonstrate an algorithm that allows refining PAM images based on numerically calculated detector PSF.

Photoacoustic microscopy [1] (PAM) is based on using focused ultrasonic detectors with high numerical aperture (NA), which allows receiving photoacoustic signals from a limited spatial region defined by transducers' focal spot. In this way, the area of possible locations of sources is determined by the focal zone of ultrasonic detector that is a distinctive feature of photoacoustic microscopy. Therefore, PAM is a simple approach to provide 3D photoacoustic imaging without reconstruction. Application of focused detectors with high NA also reduces the requirements for the laser illumination, since it suffices to illuminate a limited spatial region.

However, finiteness of focal spot size leads to blurring of resulting PAM images. Therefore, with the use of reconstruction algorithms it is possible to improve accuracy of the obtained pictures. Every time diagnosing biological tissues with a large number of inhomogeneities it is needed to know if this part of the reconstructed image is real or false (artifact), arising due to the nature of wave propagation in the medium or to the characteristics of ultrasonic transducers. Thus, solving the problem of elimination of artifacts associated with finite aperture antennas is a quite important task. It is possible to improve PAM images of initial absorption distribution finding the point spread function (PSF).

In this presentation we will demonstrate an algorithm that allows refining PAM images based on numerically calculated transducer PSF. In order to model photoacoustic pulses from point-like absorbers registered by the surface of a spherically focused transducer with focal distance $F = 8$ mm, numerical aperture $NA = 0.56$ and aperture $a = 6$ mm (Fig. 1, a), we used K-wave toolbox [2]. All calculations were made on spatial grid 126 by 126 by 126 points (or 18 by 18 by 18 mm) with the number of temporal points 351 (or 10 μ sec). Transducer's central frequency 5.25 MHz was chosen to speed up the calculation time that was rather low in comparison to frequencies used in PA microscopes [1, 3]. However, at the moment we are only interested in testing numerical methods for PAM artifacts elimination.

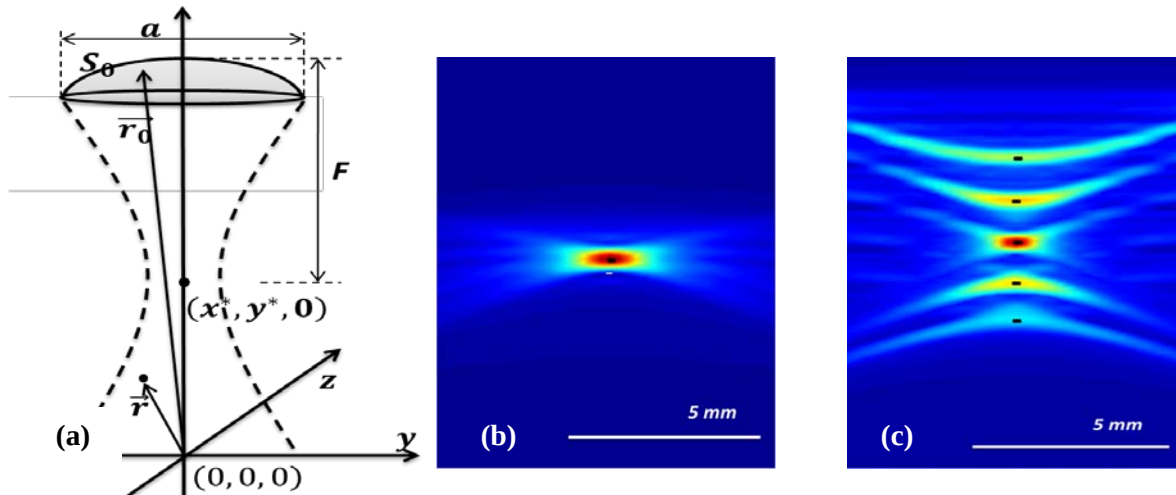


Fig. 1. Model B-scans obtained by photoacoustic microscope. (a) the scheme of model experiment (b) model B-scan of an infinitely thin thread oriented along Z-axis and placed at focal distance F from detector surface in media without acoustic attenuation (c) model B-scan of 5 infinitely thin threads oriented along Z-axis and placed with 1 mm spacing in tissue-like media with $1\text{dB/cm/MHz}^{3/2}$ acoustic attenuation

To obtain model PAM images we defined PA sources as thin threads oriented along the Z-axis in some positions determined by coordinates (X, Y) and then moved the focused detector along the Y-axis with one grid point step (which corresponds to 140 μm), obtaining independent A-scans at each particular detector position. A B-scan image of the thread placed in the center of transducer focal zone that consists of 20 A-scans is shown in Fig. 1, b. It is clearly seen how the finite size of the detector blurs the real image of a thread placed at $x = 9$ mm. A B-scan image obtained for a medium with absorption of additional threads placed above and below transducer focal spot at $x = 7$ mm, $x = 8$ mm, $x = 10$ mm, $x = 11$ mm is presented in Fig. 1, c, where one can see that blurring around real position of such threads expands even wider.

For improvement of the PAM images (Fig. 1, b, c) we proposed the reconstruction algorithm. First, we showed that absorption distribution, PA signals $p_0(x^*, y^*, \bar{t})$ measured by detector spherical surface, and effective Green function (transducer PSF) for our problem are related by the Fredholm equation of the first kind (note that the symmetry along the Z-axis allowed us to speed up the calculation without scanning along the Z-axis):

$$p_0(x^*, y^*, \bar{t}) = \iint_{S_{\text{foc}}} dx_s dy_s G_{\text{eff}}(x_s, y_s, x^*, y^*, \bar{t}) A(x_s, y_s). \quad (1)$$

We proposed a method of reconstructing absorption distribution based on $p_0(x^*, y^*, \bar{t})$ and $G_{\text{eff}}(x_s, y_s, x^*, y^*, \bar{t})$. Numerical calculation of $p_0(x^*, y^*, \bar{t})$ and $G_{\text{eff}}(x_s, y_s, x^*, y^*, \bar{t})$ allows expressing (1) in matrix form: $P = GA$, where matrix A (distribution of optical absorption coefficient) must be non-negative. Taking into consideration the features of A in the obtained system of linear equations, we used the NNLS method of least squares for solution of the non-negative problem [4].

After reconstruction we obtained ideal pictures with single point-like sources. Then we started adding noise to PA signals $p_0(x^*, y^*, \bar{t})$ as real photoacoustic measurements always have noise background. We have chosen noise values uniformly distributed in the range $[-p_{\text{max}}, p_{\text{max}}]$ where $p_{\text{max}} = \max\{p_0(x^*, y^*, \bar{t})\}$. In Fig. 2 one can see how the quality of the reconstructed image degrades with addition of noise. It is also worth noting that the NNLS method is noise-resistant. Only addition of 1000% of noise blurs the reconstructed image; it becomes impossible to determine real position of the source (Fig. 2, c).

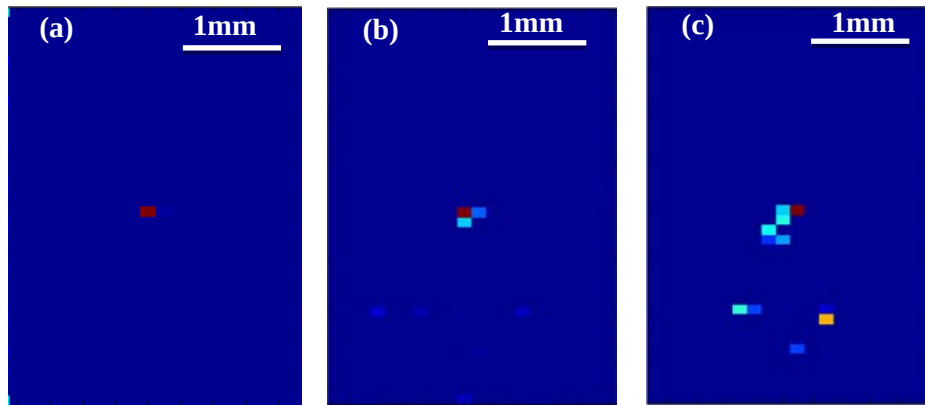


Fig. 2. Reconstructed photoacoustic images obtained by NNLS algorithm (a) with addition of 40% noise to PA signals; (b) with addition of 280% noise to PA signals; (c) with addition of 1000% noise to PA signals

Acknowledgements

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INFLUENCE OF IRRADIATION ON THE OXYGENATION OF EXPERIMENTAL TUMOR ESTIMATED BY DIFFUSE OPTICAL SPECTROSCOPY

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Abstract. We used Diffuse Optical Spectroscopy to investigate the dynamics and variation mechanisms of the oxygenation level of an experimental tumor over a period from 24 to 96 hours following its single-dose irradiation. Plyss lymphosarcoma oxygenation decreased 24 hours after treatment because of the increase in the concentration of deoxyhemoglobin in the tumor tissue. In 48 hours moderate tumor reoxygenation was detected and oxygenation reached a control level 96 hours after irradiation. At the root of the reoxygenation process was the transient elevation of the oxyhemoglobin level.

Tumor oxygenation and variations thereof under the effect of ionizing radiation are among the key factors influencing effectiveness of radiotherapy. Assessment of the dynamics of the oxygenation level in the tumor zone allows choosing optimal conditions of treatment and plays an important role in creating alternative modes of radiation therapy [1]. To study the oxygen delivery/consumption balance and the role of the microcirculation response in the variations of tumor oxygenation under the action of irradiation the Diffuse Optical Spectroscopy (DOS) was used. DOS makes possible the noninvasive determination of tissue oxygen status based on information on the local changes in the optical parameters (absorption and scattering), and visualization of metabolic processes in the region of interest. DOS allows reconstructing two-dimensional distribution of the main tissue chromophores that characterize the processes of oxygen supply (oxyhemoglobin) and oxygen consumption (deoxyhemoglobin), as well as the blood oxygen saturation level (StO_2) that indirectly reflects tissue oxygenation. The goal of the study was to investigate the experimental tumor oxygen status using DOS *in vivo* during tumor growth and under radiation therapy.

The experiments were carried out on white outbreed rats. Tumor model of Plyss lymphosarcoma (PLS) [2] characterized by rapid growth and early occurrence of necrotic areas was used. The tumor was transplanted subcutaneously into the right lower third of the abdominal wall. Experiments on DOS were performed on the experimental setup with parallel plane geometry created at the Institute of Applied Physics RAS (Nizhny Novgorod, Russia). Three lasers at the wavelengths of 684 nm corresponding to the high absorption of deoxygenated hemoglobin, 850 nm corresponding to high absorption of oxyhemoglobin, and 794 nm at which absorption coefficients of oxygenated and deoxygenated hemoglobin are identical have been used as light sources. Images were obtained by synchronous «step by step» moving of the source and the detector located along the sagittal axis from the opposite sides of the studied subject.

Prior to investigation the animals were anaesthetized and placed into a cuvette filled with an immersion liquid of known optical parameters [3]. The distribution of oxyhemoglobin (HbO_2), deoxyhemoglobin (HHb), and blood oxygen saturation ($StO_2 = [HbO_2]/[HbO_2 + HHb] \times 100\%$) [4] in tissues was numerically reconstructed. Tumors were irradiated with a single dose of 10 Gy (Co^{60}) unit. The first DOS procedure was on the 7th day of tumor growth directly before irradiation. DOS procedures were repeated every 24 hours after irradiation for 96 hours. DOS imaging of a control group was performed in the same time after inoculation. A direct measurement of pO_2 of tumor tissue was used as a method of verification of DOS results in three irradiated animals [5]. After scanning, two untreated animals on the 7th and the 9th day and two irradiated on the 8th and the 9th day after inoculation (24 and 48 hours after irradiation respectively) were sacrificed and tumors were dissected for the histological study. The total of 21 tumor-bearing animals (13 irradiated and 8 untreated) were monitored.

To analyze DOS results, we used the ratios (mean values and standard deviations) of the HbO_2 , HHb, and total hemoglobin (tHb) concentrations and StO_2 in the tumor region and the contralateral region of the animals. Spearman's rank correlation coefficient (r) was calculated in order to establish correlation between the absolute StO_2 values and the results of direct pO_2 measurements in the tumor.

Comparison between the tumor tissue oxygenation data obtained by the DOS and direct pO_2 measurements demonstrated high correlation coefficient between two methods ($r = 0.88$, $p < 0.01$). Our investigation demonstrated that radiation-induced oxygenation changes of Plyss lymphosarcoma were of a biphasic character. Recorded 24 hours after irradiation was a lowered blood oxygen saturation level as compared both with its initial values ($p < 0.05$) and with that in the untreated animals (Fig. 1). This lower level owed to the increase in the concentration of HHb in the tumor tissue compared to its initial level ($p < 0.05$). As compared with that in the control group, the differences were statistically significant as well ($p < 0.01$). A reduced HbO_2 concentration ($p < 0.05$) was observed at the same time. From this point the oxygenation level of the tumor was observed to grow higher, its maximum being reached 48 hours after treatment. Noted at this time was a statistically significant increase in the StO_2 of the tumor compared to that in the untreated animals, which persisted for the next 24 hours ($p < 0.01$). At the root of the reoxygenation process was the elevation of the HbO_2 as compared to its initial values ($p < 0.01$) and to that in the untreated animals ($p < 0.01$).

At the same time, HHb concentration was observed to revert. Hereafter the StO_2 gradually lowered to reach its control values within 96 hours of irradiation. Total hemoglobin concentration remained unchanged in comparison with both the initial and the control level. The dynamics of the concentrations of the different forms of hemoglobin reflects the changes in the condition of tumor vascular bed revealed through histological examination.

The results obtained show it is the perfusion disorders which can cause the tumor oxygenation level to fall that play the chief role during the first phase of the radiation-induced alteration of the oxygenation status of the tumor model under investigation. Considering the contradictory character of the investigation results obtained on different tumor models, one can suppose different directions of the tumor vascular bed reaction to radiation therapy in each particular case. This fact should be taken into consideration in developing nonstandard fractionation regimens and radiomodifying treatments based on the oxygen effect.

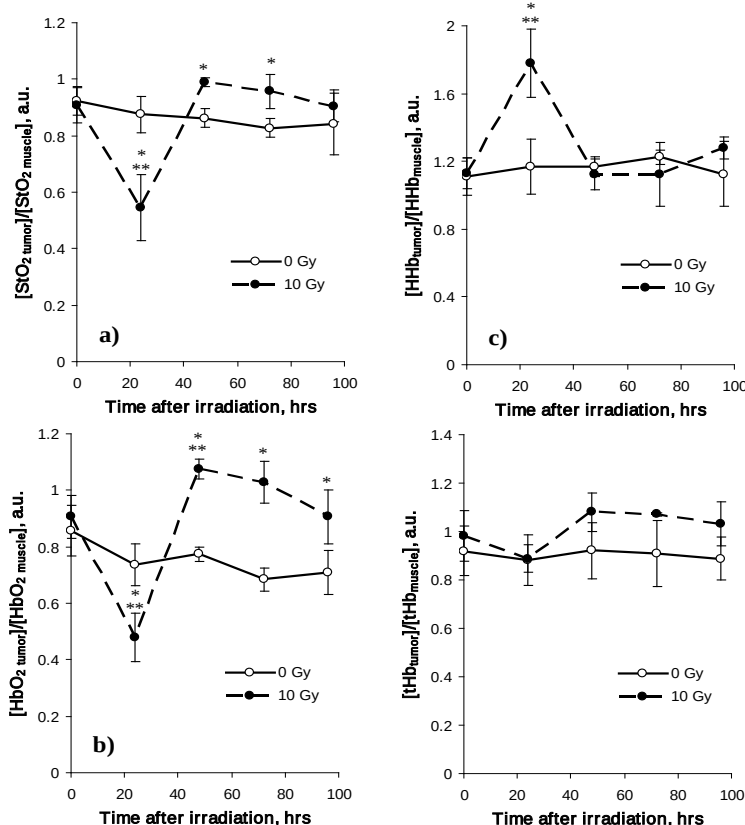


Fig. 1. Dynamics of HHb, tHb и HbO_2 of PLS after irradiation in comparison with non-irradiated tumors

*Differences are statistically significant between two groups ($p < 0.05$) based on t-test;

**Differences are statistically significant as compared to the initial level ($p < 0.05$) based on t-test.

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NEW TOOLS TO ASSESS BREAST CANCER TUMOR MARGINS: OCT NEEDLE PROBES AND OPTICAL COHERENCE ELASTOGRAPHY

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Abstract. Breast cancer is an ongoing major health issue worldwide. Our group has been investigating several strategies based on optical coherence tomography (OCT) to improve breast cancer detection and treatment. Our OCT "microscope in a needle" guided to the site of a tumor under ultrasound imaging has shown the capacity to identify tumor margins with microscopic resolution in freshly excised breast tissue. We are also pursuing applications in breast cancer with the newly emerging technique of optical coherence elastography, in both 3D and needle-based formats, with promising results.

Motivation: In the USA, breast cancer is the most common cancer in women, with 288,000 new cases diagnosed each year [1] and 40,000 deaths. In Australia, it is the leading cause of death in women aged 25-64 years (15% of all deaths) [2]. Surgical removal of the malignant tissue forms a central component of treatment. Although pre-operative imaging provides a useful guide, accurate identification of the extent of malignancy is difficult during surgery – the surgeon is, in effect, "cutting blind". In 34% of patients undergoing breast-conserving surgery, tumor removal is insufficient: the margins of the removed tissue contain malignant tissue or an insufficiently wide border of cancer-free tissue [3]. The result is increased risk of local recurrence with 26% of patients requiring further surgery [3]. Improving the success of breast-conserving surgery will benefit breast cancer patients by reducing the incidence of further treatment, as well as reducing health-care costs.

OCT Microscope in a Needle: By incorporating an OCT imaging probe into a needle, it is possible to locate the needle tip deep in tissues and record a three-dimensional OCT image. We typically achieve this with ultrasound guidance of a needle with an overall size range of 22-30 G (720-310 μm outer diameter). Three-dimensional OCT scans can be recorded by rotating the needle, which has a side-directed optical beam, whilst translating it in the tissue [4]. We have also recorded two-dimensional scans by "push-pull" linear scanning needles to achieve higher speed imaging of dynamic processes [5].

Recently, we have demonstrated a range of technical advancements in OCT needle probes that advance their imaging and functional performance. Our advancements include: three-dimensional OCT imaging [4], confocal microscopy for resolution superior to OCT in needles [6], ultrathin needle probes [7], extended depth of focus probes [8], use of mechanical contrast – needle elastography [9], hand-held scanning using magnetic-sensor navigation [10], anastigmatic, high-sensitivity capillary needle designs [11], and the combination of OCT with fluorescence in a needle [12].

Beyond technical innovation, we have begun applying microscope-in-a-needle imaging to a wide variety of tissues and pathologies in animals and humans. Whilst our primary focus is on breast and lymph nodes (for cancer) [13, 14], we have also performed needle OCT imaging on lungs (for animal models of chronic obstructive pulmonary disease) [4, 5] and skeletal muscle (for muscular dystrophy).

Optical Coherence Elastography: The exquisite sensitivity of phase-sensitive optical coherence tomography presents new opportunities in optical coherence elastography (OCE) for the measurement and imaging of tissue mechanical properties on the microscopic scale [15]. We have been working to develop OCE methods and techniques with a strong focus on breast cancer. To make OCE on human subjects feasible, we designed an annular piezoelectric loading transducer, through which we could simultaneously image, thus, enabling the first *in vivo* dynamic optical coherence elastography [16, 17]. We extended this to three dimensions [18] and to frequency-swept acoustic loading [19], in collaboration with Stephen Boppart at the University of Illinois at Urbana-Champaign. Since then, we have developed further important improvements to the signal processing methods [20, 21] and framed an appropriate theoretical basis for benchmarking OCE methods [20]. We have also been developing OCE methods utilizing needle probes [9]. We

have begun to apply 3D-OCE and needle OCE in breast cancer, with encouraging results to be reported at the conference.

Acknowledgements

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MONTE CARLO SIMULATION OF OPTICAL BRAIN SENSING IN DIFFERENT GEOMETRIES

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Abstract. We report on development of novel Monte Carlo technique for simulation of light transport in heterogeneous media based on triangulated surfaces approach. The code was employed to model near infrared spectroscopy (NIRS) sensing of human brain in several geometries corresponding to head-mimicking phantoms (planar and spherical) and real head geometry. The fractional contribution of photons reaching the cortex layer into NIRS signal was studied. Differential pathlength factor (DPF) was evaluated for all geometries and the influence of the considered geometry on DPF values was analyzed.

Advancement in novel optical techniques for biomedical diagnostics and treatment requires accurate description of light propagation in biological objects. Application of theoretical approximations is limited by diversity of optical parameters of biotissue layers and complex boundary conditions. As an alternative, numerical methods for modeling light transport in complex media can be applied. One of them is Monte Carlo based statistical method which has recently gained several advantages from innovations in technology and methods. Implementation of CUDA architecture into conventional Monte Carlo code resulted in calculation speed-up by several orders of magnitude using graphical processing units compared to a single CPU. Applying mesh based approach in simulation of light interaction with the boundary of an arbitrary shape allowed adaptation of Monte Carlo algorithm for simulation of light propagation in complex biological objects like human head whose realistic geometry can be set by individual MRI data.

We report on development of adapted Monte Carlo based algorithm and proper code for simulation of light propagation in turbid media with complex geometry aimed at simulation of near infrared spectroscopy (NIRS) signal in noninvasive brain sensing. Simulation is performed for planar, spherical and realistic head geometry with account for the major anatomic structures of human head (scalp, fat layer, skull, cerebrospinal liquid, grey matter, and white matter). The thickness of the layers was chosen based on anatomic data obtained from several MRI images of human head. Optical properties of all layers for the probing wavelengths of 650, 830 and 915 nm were summarized from the literature [1, 2]. Prior to simulations, the validation of the algorithm was performed by comparing the results of simulation in semi-infinite geometry to the analytic results obtained from diffusion theory. Performance of the developed code was checked for shared memory systems and the efficiency of parallelization of 75% was achieved for all three considered geometries of the head model. Using the developed code we simulated the

characteristics of NIRS signal obtained at various source-detector separations (D_{sd}) of a typical two-position NIRS system.

Figure 1 shows spatial distribution of photons which form NIRS signal at the D_{sd} of 40 mm in the three considered geometries. The calculation demonstrates that for a given D_{sd} sufficient fraction of the detected photons propagate through the cortex area and thus carry information on the functional activity of the brain. Quantitative estimation of such fraction has been performed for various values of D_{sd} . The results for all geometries are demonstrated in Fig. 2 which shows that the separation at which the photons coming from the cortex start to dominate is about 20, 25 and 30 mm for the wavelengths of 660, 830 and 915 nm, respectively, and the outcome is almost insensitive to the chosen head geometry.

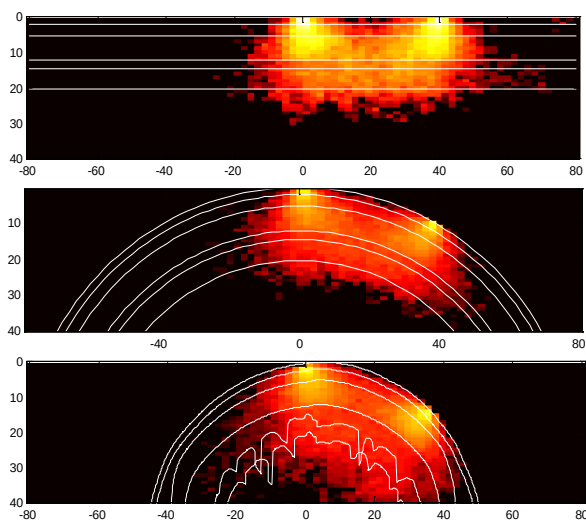


Fig. 1. Spatial distribution of photons which form NIRS signal at the wavelength of 830 nm for plane (top), spherical (mid) and realistic (bottom) head geometries. $D_{sd}=40$ mm

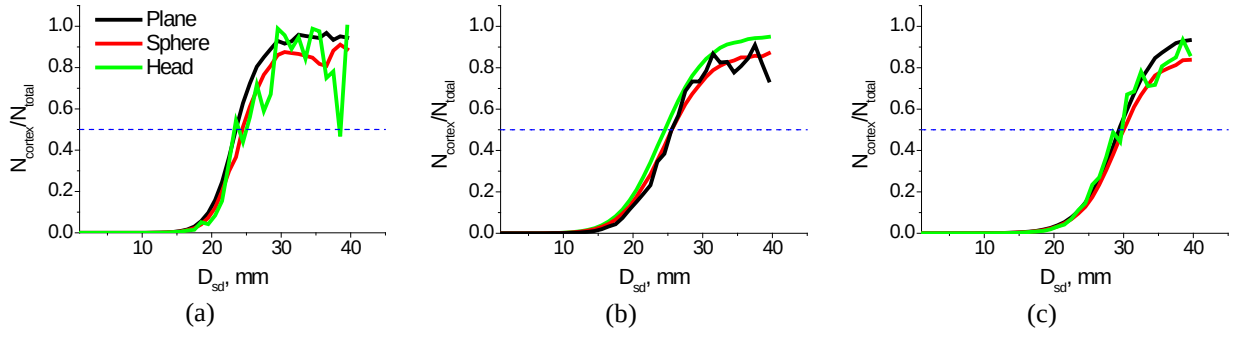


Fig. 2. Relative fraction of NIRS signal that reaches the cortex area in three geometries as the function of source-detector separation: (a) 660 nm; (b) 830 nm; (c) 915 nm

Noninvasive estimation of brain activity is based on the dynamics of oxy- and deoxyhaemoglobin within different regions of cortex. Concentration of these chromophores is estimated from NIRS signal based on so-called modified Beer-Lambert law which relates the registered intensity to the average absorption coefficient of the studied region and the average pathlength of diffusive light [3]. The crucial point of modified Beer-Lambert law application is the differential pathlength factor (DPF) which is the ratio of average pathlength of multiply scattered photons to the value of D_{sd} . We performed numerical analysis of DPF values in different geometries employing the developed Monte Carlo code and compared the obtained values with the theoretical estimates of DPF calculated for semi-infinite uniform medium which is characterized by optical properties of scalp and gray matter. The behavior of DPF dependence versus the D_{sd} is quite complex and differs from that in uniform medium, though showing no considerable difference for the three studied head geometries (Fig. 3). However, comparison shows that for the D_{sd} values corresponding to the dominating fraction of photons that reach cortex the values of DPF obtained from numerical simulations tend to those calculated analytically for the medium mimicking uniform gray matter. This tendency is universal for all selected wavelength and head geometries and can be used for fast estimations of DPF in practical tasks.

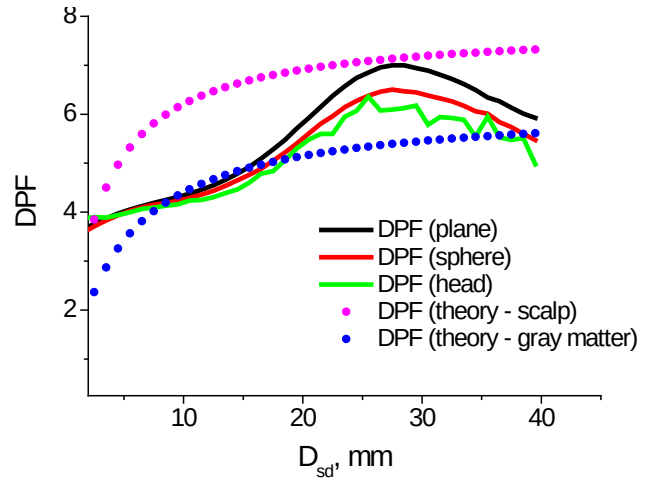


Fig. 3. Differential pathlength factor simulated for three geometries as the function of source-detector separation (wavelength 830 nm)

Acknowledgements

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3D BROADBAND DIGITAL HOLOGRAPHIC MICROSCOPY

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Abstract. The work is devoted to development of digital microscopy methods for visualization of internal structure of objects, including biological ones. Planar microscopic objectives were used in a broadband holographic setup. A lateral resolution of $1 - 3 \mu\text{m}$ and spectrally conditioned longitudinal resolution were obtained. Two different methods of complex hologram formation – consecutive and parallel were tested and their merits and drawbacks were considered. Essential improvement of the algorithm of input data processing and an increase in the calculation speed in a wide-aperture mode, without a low-angle approach, was attained.

A version of a broadband holographic setup using a planar microscopic objective was developed. The optical scheme on the basis of the Mach-Zehnder interferometer (fig. 1) was constructed.

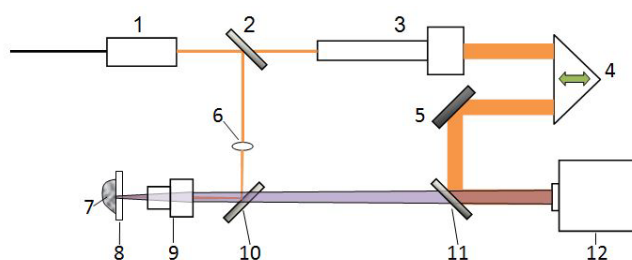


Fig. 1

The beam, emergent from collimator 1, was divided by semi-transparent mirror 2 into reference and object beams. Further, the object beam was broadened with telescope 3 to illuminate evenly a full surface of CCD matrix 12. Prism 4 was inserted for adjustment of the phase delay and recording holograms in complex form. An additional lens 6 was inserted on the path of the object beam for formation together with microscopic objective 9 of a telescope to reduce the section of the beam illuminating the object. The investigated object 7 was placed behind a thin cover glass 8 with the top surface adjusted so as to coincide with the focal plane of objective 9. As a result, an enlarged image formed on the top surface of the cover glass 8 by the radiation scattered from the 3D object was transferred to the CCD matrix surface through semi-transparent mirrors 10 and 11. The method of broadband digital holography, when a 3D image of an object is formed as a result of recalculation of a set of two-dimensional holograms obtained at different wavelengths at retuning of the radiation source was employed.

Two objectives – LOMO PLAN 9x0.2 and LOMO PLAN 3.5x0.1 were tested in the setup. The lateral resolution of $1.5 \mu\text{m}$ and $3 \mu\text{m}$, respectively, was obtained with the use of these objectives. The results of drosophila visualization are presented in fig. 2. Its eye with a facet structure is shown in fig. 2(a), and the whole little body in fig. 2(b).

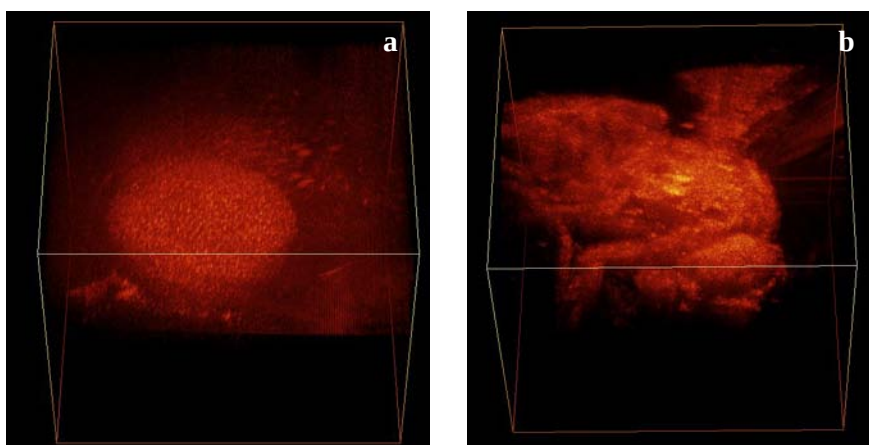


Fig. 2

Spectrally conditioned longitudinal resolution of 10 μm (effective width of retuning was 30 nanometers, central wavelength was 0.85 μm) was obtained.

Two different methods of formation of complex holograms – parallel and consecutive were tested. The parallel method demands only one shot for formation of a complex hologram, whereas the consecutive method needs three shots. Also, the parallel method is less sensitive to parasitic vibrations. But this method provides a smaller dynamic range and two-times smaller lateral resolution; in addition, exact adjustment of the angle of incidence of the reference wave (by means of mirrors 5 and 11, fig. 1) is demanded. In the conditions of compact setup, accurate measurement of the angle of incidence is strongly complicated; therefore, we developed a method for its exact adjustment using the phase histogram of the received signal.

The algorithm of input data processing has been improved significantly. A 3D image is reconstructed by the method of decomposition in the matrix plane $S(x,y,0)$ into transversal spatial harmonic as k_x, k_y : $FFF2d_C(k_x, k_y)_0$. Phase correction of this Fourier-transformation is made for field reconstruction at distance Z from the matrix:

$$FFF2d_C(k_x, k_y)_Z = FFF2d_C(k_x, k_y)_0 \cdot \exp(-i \cdot Z \cdot \sqrt{k^2 - k_x^2 - k_y^2}) \quad (1)$$

with subsequent inverse Fourier-transformation. At wide-angle reception, expression (1) cannot be reduced to 3D Fourier transform. Therefore, this operation needs to be done for a full visualized volume (for each plane for all discrete values of Z) at each wavelength. The result obtained is then summarized for all wavelengths. There is, however, another way. The spectral transformation $FFF2d_C(k_x, k_y)_0$, in fact, sets in the Fourier space a surface of values for a true 3D Fourier-transformation. This surface is described by the following formula:

$$k_z = \sqrt{\frac{2\pi^2}{\lambda} - k_x^2 - k_y^2}, \quad (2)$$

where λ is the current length of the wave. But with a discrete set of values and, respectively, a discrete coordinate grid, the transcendental value (2) does not coincide with the grid. However, any spectral element with amplitude A and carrier frequency k_z that does not coincide with any discrete value of the grid may be represented as a Fourier-transform of the function $A \cdot e^{i \cdot Z \cdot k_z}$. A typical form of this function (amplitude and phase) is depicted in fig. 3.

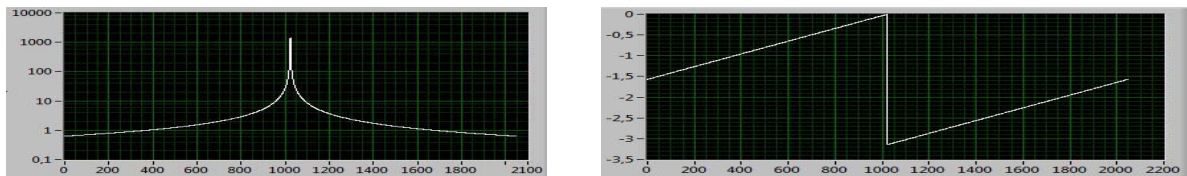


Fig. 3

This function can be multiplied by $E(k, k_z) = e^{-(k - k_z)^2 / dk^2}$ and constrained by a number of points in the dk neighborhood symmetric relative to k_z without essential loss of accuracy of representation. Further, we summarize in the k -space segments of these functions along the k_z axis with magnitudes defined by $FFF2d_C(k_x, k_y)_0$ for each wavelength. Next, we make the inverse 3-D Fourier-transform. This procedure of reconstructing a 3D image is much faster.

Acknowledgements

The work was supported by the RFBR grant 09-02-00650-a and State Contract 02.740.11.0516.

LIFETIME IMAGING WITH NEAR-INFRARED FLUOROPHORES

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Abstract. Near-infrared (NIR) dyes are used as fluorescence markers in small-animal imaging and in diffuse optical tomography of the human brain. In these applications it is important to know whether the dyes bind to proteins or other tissue constituents, and whether their fluorescence lifetimes depend on the targets they are bound to. We upgraded existing confocal TCSPC FLIM systems with NIR lasers and NIR sensitive detectors. We tested a number of NIR dyes in biological tissue. Some of them showed clear lifetime changes depending on the tissue structures they are bound to. We therefore believe that NIR FLIM can deliver supplementary information on the tissue constitution and on local biochemical parameters.

Introduction

At first glance, TCSPC FLIM [1] at NIR wavelengths should neither be a problem on the detection nor on the excitation side: FLIM detection in the 700 to 900 nm range can be achieved by bh HPM-100-50 hybrid detectors or by PMC-100-20 PMT modules [2]. Picosecond diode lasers for the red and near-infrared range are available with 640 nm, 685 nm, and 785 nm, the Intune of the Zeiss LSM 710 can be tuned up to 645 nm, and super-continuum lasers with acousto-optical filters deliver any wavelength from the visible range to more than 1000 nm. Unfortunately, there are a few pitfalls in the optics of the laser scanning microscopes: because the fluorescence is excited via a one-photon process confocal detection must be used to obtain clear images of tissue samples. That means the internal beamsplitter of the scan head must reflect the excitation light towards the microscope lens, and transmit the fluorescence back from the lens to the detectors. Neither a normal confocal microscope, nor a multiphoton microscope has the right beamsplitter: The transition wavelengths of the beamsplitters of a confocal microscope are too short, and the transmission range of the beamsplitter of a multiphoton microscope is at the wrong side of the excitation wavelength. The ideal solution would be a main dichroic beamsplitter that reflects the laser up to about 700 to 800 nm, and transmits the fluorescence at longer wavelengths. Several such beamsplitters would be required for dyes of different absorption and emission wavelengths. However, replacing a main dichroic beamsplitter in the scan head of a laser scanning microscope is not easy. The problems can be solved by using a wideband beamsplitter in the scan head. The loss in the excitation path can be compensated by increasing the laser power at the input of the scanner. The loss in the detection path can be kept at a tolerable level by using a beamsplitter that has a transmission of 60 to 80%. Wideband beamsplitters are available in the bh DCS-120 WB confocal FLIM system and in the Zeiss LSM 710 family microscopes. In these systems, the only requirement for NIR FLIM is that a suitable excitation source and a confocal output to the FLIM detector be available.

Results

A pig skin sample stained with 3,3'-diethylthiatricarbocyanine is shown in Fig. 1, left. The sample was immersed in a solution of the dye for 10 minutes. The image was recorded by a Zeiss LSM 710 NLO system and a bh Simple-Tau 150 FLIM system. The Ti:Sapphire laser was used as a one-photon excitation source. The laser wavelength was 780 nm. The 80/20 beamsplitter of the LSM 710 scan head was used, and the fluorescence selected by a 800 nm long-pass filter in front of an HPM-100-50 detector attached to the confocal port. The lifetime is short where the tissue was exposed to high dye concentration, and longer inside the tissue. This may be a pure concentration effect due to aggregation of the dye. The lifetime changes in Fig. 1, left, may therefore not reflect any biologically relevant parameters. Even so, the long excitation and detection wavelength results in high contrast images as they are not normally obtained by confocal imaging of thick tissue. An image of a sample immersed in a solution of 3,3'-diethylthiatricarbocyanine for 24 hours is shown in Fig. 1, right. This image was recorded by a bh DCS-120 WB system at 650 nm excitation wavelength. There is not only excellent contrast in the image, but also different fluorescence lifetime in different tissue structures. Whether the lifetime variation is a result of the long immersion time, what the mechanism of the lifetime variation is, and to which tissue parameters the lifetime reacts requires further investigation.

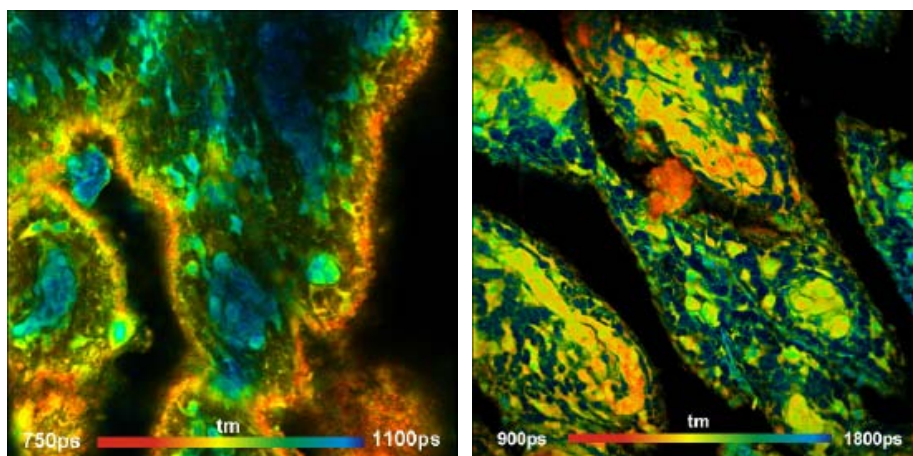


Fig. 1. Pig skin samples stained with 3,3'-diethylthiatricarbocyanine. *Left:* Zeiss LSM 710 NLO, Ti:Sa laser, one-photon excitation, excitation at 780 nm, detection from 800 nm to 900 nm, HPM-100-50 detector. *Right:* bh DCS-120 WB confocal FLIM system, excitation 650 nm, detection 680 nm to 900 nm, HPM-100-50 detector

Fig. 2, *left*, shows a pig skin sample stained with methylene blue. As can be seen from the lifetime image and from the decay curves (Fig. 2, *right*) methylene blue delivers distinctly different decay times depending on the tissue structures it binds to. Both the lifetimes and the amplitudes of the decay components change. The exact mechanism of the lifetime changes is not known. However, methylene blue shows changes in its absorption spectrum on pH variation. It can be expected that these are accompanied by lifetime changes. More important, it is known that methylene blue is a redox indicator. It is thus possible that the changes originate from variation in the redox potential of the proteins. If this is correct, methylene blue could be a highly potent marker for the tissue state.

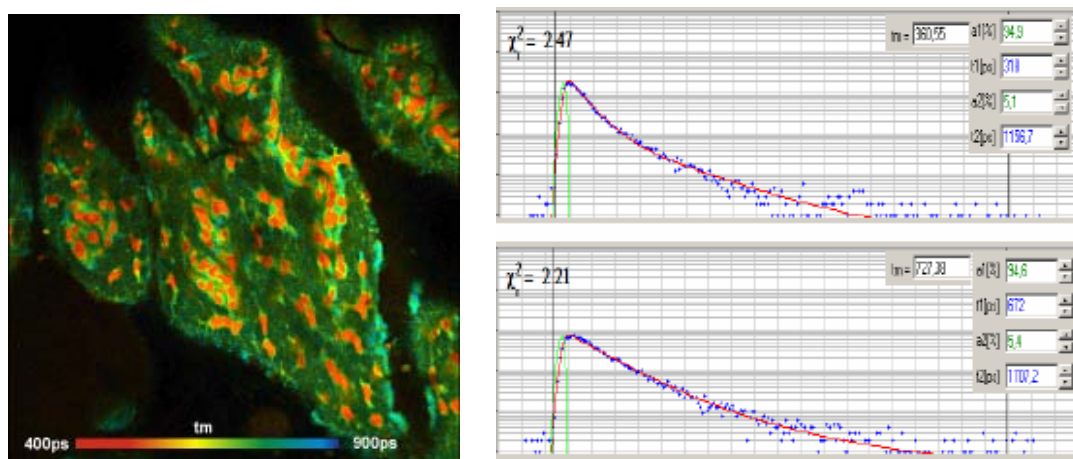


Fig. 2. Pig skin stained with methylene blue. *Left:* Lifetime image, double-exponential model, amplitude-weighted lifetime. *Right:* Decay curves in 3x3 pixel areas. *Top:* From red spots. *Bottom:* From green areas. DCS-120 WB system, 640 nm ps diode laser

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FLUORESCENCE LIFETIME-TRANSIENT EFFECTS RECORDED BY LINE SCANNING

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Abstract. We present a technique that records transient effects in the fluorescence lifetime of a sample with spatial resolution along a one-dimensional scan. The technique is based on building up a photon distribution over the distance along the scan, the experiment time after stimulation of the sample, and the arrival times of the photons after the excitation pulses. The maximum resolution at which lifetime changes can be recorded is given by the line scan time. With repetitive stimulation and triggered accumulation transient lifetime effects can be resolved at a resolution of about one millisecond.

Introduction

Fluorescence lifetime imaging (FLIM) by multidimensional TCSPC is based on raster-scanning a sample, detecting single photons of the fluorescence light emitted, and building up a photon distribution over the coordinates of the scan area, x and y , and the arrival times, t , of the photons after the laser pulses. For multi-wavelength FLIM the wavelength of the photons is added as an additional dimension. The results can be interpreted as an array of pixels, each containing a large number of time channels for consecutive times after the excitation pulses [1]. Transient effects in the fluorescence lifetime can be recorded by time-series FLIM. Subsequent FLIM recordings are performed, and the data saved into consecutive data files. Time-series of FLIM images can be recorded at surprisingly high rate, especially if readout times are avoided by dual-memory recording. Nevertheless, each step of a time series requires at least one complete x - y scan of the sample. With the typical frame rates of fast galvanometer scanners lifetime changes can be recorded at a maximum resolution on the order of 100 ms to 1 second. Lifetime changes faster than that can be recorded by single-point measurements, as has already been demonstrated in [1]. However, single-point measurements do not deliver information about the spatial distribution of the effects observed.

Technical Solution

A solution to spatially-resolved transient recording is, again, provided by multi-dimensional TCSPC. The approach is illustrated in Fig. 1. Figure 1, *left*, shows the photon distribution built up by normal TCSPC FLIM. It is a distribution of photon numbers over x , y , and t . In Fig. 1, *right*, one spatial coordinate (y) has been replaced with an 'experiment time', T . The experiment time, T , is the time after a stimulation of the sample, or after any other event that is expected to be temporally correlated to a lifetime change in the sample. X is the distance along a spatially one-dimensional scan.

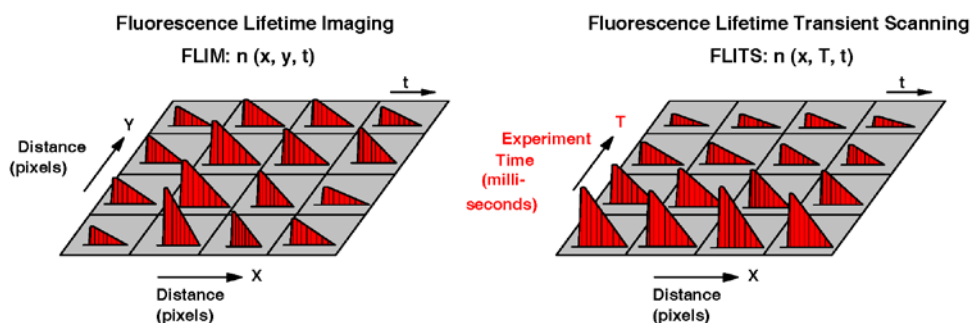


Fig. 1. *Left:* Photon distribution built up by standard FLIM.
Right: Photon distribution built up by fluorescence lifetimetransient scanning

As long as the stimulation occurs only once the recording process appears simple: The sequencer of the TCSPC module starts to run with the stimulation, and puts the photons in consecutive time channels along the T axis. The result is a time-series of line scans. Obviously, the resolution in T is limited by the period of the line scan only, which is about 1 ms for the commonly used scanners.

Results

To demonstrate fluorescence lifetime-transient scanning we used the ‘chlorophyll transients’ that occur when a live plant is exposed to light. Upon illumination, the fluorescence lifetime (and intensity) first increases. The increase happens within a few milliseconds or tens of milliseconds. It is attributed to the progressive saturation of photosynthesis channels, and a corresponding decrease in fluorescence quenching. The increase in the fluorescence quantum efficiency is therefore called ‘photochemical transient’. Recording the photochemical transient requires periodical stimulation and acquisition of the data over a long period of time. Both the laser on-off signal and the frame clock were therefore generated by a bh DDG-210 pulse generator card. The on-off period was 1 second, the ‘on’ time within the period was 200 ms. Each turn-on of the laser initiates a photochemical transient, i.e. an increase of the fluorescence lifetime. In the laser-off period the leaf partially recovers, so that the next laser-on initiates a new transient. The total acquisition time was 40 seconds, i.e. 40 on-off periods were accumulated. The result is shown in Fig. 2.

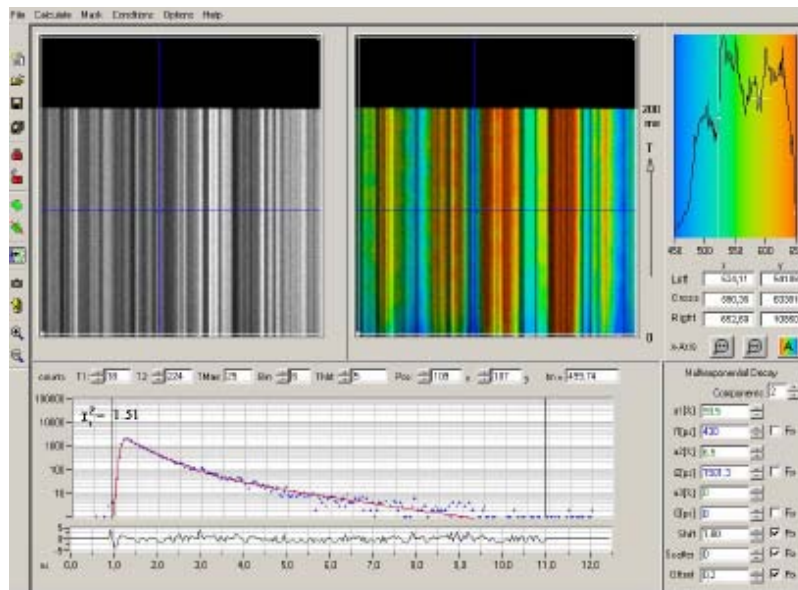


Fig. 2. FLITS image of photochemical chlorophyll transient. Horizontal: Distance along the line scanned. Vertical, bottom to top: Time, T , after the start of illumination. Line time 1 ms, 256 lines, total time interval recorded 256 ms. Laser turned on at $T=0$, laser-on time 200 ms. Left: Intensity image. Right: Lifetime image. Colour represents lifetime, blue to red corresponds to 450 ps to 650 ps. Amplitude-weighted lifetime of double-exponential fit

Summary

FLITS records fluorescence-lifetime transients at a time scale down to about one millisecond with spatially one-dimensional resolution. Technically, FLITS is obtained by line scanning and replacing the ‘frame clock’ of a TCSPC FLIM system with a trigger pulse that is synchronous with the event that stimulates the lifetime change in the sample. The technique can thus easily be implemented in confocal or multiphoton laser scanning microscopes, provided these are able to run a fast line scan. The technique works both with single-shot stimulation, or with periodic stimulation. For a given photon detection rate, the lifetime accuracy for single-shot stimulation decreases with decreasing time-channel width along the transient-time axis. This is not the case for periodic stimulation: Here the accuracy depends on the total acquisition time. Periodic stimulation is thus the key to high fluorescence-transient resolution. Potential applications of FLITS are experiments of plant physiology, electrophysiology, and Ca^{++} imaging of neuronal tissue.

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DOUBLE-PRISM CORRECTION OF SPECTROMETER FOR SD OCT

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Abstract. A detailed analysis of a single-prism corrector of nonequidistance of a grating-based spectrometer is carried out. The influence of focusing lens distortion on spectrometer nonequidistance is considered. A novel setup for optimal correction of spectrometer nonequidistance is proposed. The setup allows tuning the spectrometer to optimal correction of nonequidistance in a wide range of central wavelengths and focusing lens distortions of illumination source.

Fourier domain optical coherence tomography (FD OCT) is based on measuring optical spectrum of a sum of two interfering waves: the reference one and the wave backscattered from the object [1, 2]. The information about object's inner structure is reconstructed by inverse Fourier transform of the optical spectrum. The Fast Fourier transform (FFT) is used to obtain maximized processing speed [3]. The use of FFT implies strongly equally spaced (by argument) samples (in FD, an OCT argument is optical frequency of received light).

In real systems this equidistance is guaranteed only to limited accuracy [4, 5]. Recently a number of methods of correcting received spectra have been developed. A large class of methods is based on numerical resampling and (or) nonequidistance digital Fourier transformation. At present, these methods use a graphics processor unit for fast operation [6-8].

Unfortunately, the use of numerical methods only results in a decrease of maximum observation depth [9]. Widening of A-scan profile leads to violation of the Nyquist criterion at large observation depth. Hence, the profile of the scatterer located at a large depth cannot be properly reconstructed by numerical methods.

Another class of methods is based on optical transformation of dispersion characteristics of the spectrometer by using an additional optical element [9, 10]. The simplest way is to use a grating-prism combination. An additional prism may be used either as a correcting dispersion element [10] or as a non-dispersion element [9] correcting only geometrical characteristics of the spectrometer.

The direct use of these methods does not allow obtaining an ideal universal setup for light sources with close but different central wavelengths. First, the nonequidistance compensation in the setup proposed in [9] depends on both, prism angle and its tilt angle. Depending on the source central bandwidth, this pair of the parameters corresponds to optimal correction of each spectral component position in the photodiode array. It allows reconstructing the profile of any surface without FWHM widening in a discrete system (the nonequidistance is less than 0.2%) [9].

As OCT devices use logarithmic scale for visualization of the object structure, the visible profile widening in the case of the nonequidistance of about 0.2% becomes significant [11]. So, the criterion of nonequidistance should be at least 10 times tighter. Areas of less than 0.02% nonequidistance for a single prism setup do not overlap for sources with close central wavelengths of 1250 nm and 1310 nm.

The second reason is the dependence of the optimum area location on focusing optics aberrations such as distortion. In conventional focusing lenses this parameter is about units of percent, which will dramatically increase the nonequidistance level of 0.2% necessary for 512-photosample recording [9]. Evidently, taking into account the lens distortion complicates calculation of the full system. We use a specially designed distortion-free lens to avoid this problem, but this setup is very vulnerable to assembling and tuning accuracy, so a possibility of lens distortion correction by prism compensator should be provided.

All the mentioned above shows that it is necessary to create a new prism every time when light source change is needed for optimal compensation of spectrometer nonequidistance.

We propose a novel prism compensator that uses a dual prism setup as is shown in Fig. 1, with very small dispersion coefficients of prism material.

The setup can be tuned to optimal state for a wide range of illumination source central wavelengths and focusing lens distortions.

Using two separated prisms one can construct an optical device similar to a single prism with another prism angle. By tuning the angle between the prisms and tilting the construction relative to the

diffractive grating surface it is possible to optimally correct the spectrometer nonequidistance in a wide range of source central wavelengths and focusing lens distortions.

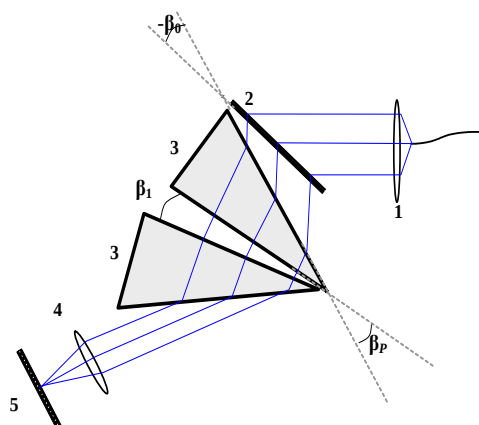


Fig. 1. A model of improved partially equidistant spectrometer. 1 – collimating lens; 2 – diffraction grating; 3 – additional prism; 4 – focusing lens; 5 – linear CCD array

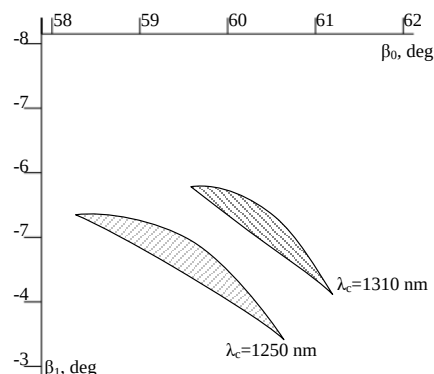


Fig. 2. Areas of less than 0.02% nonequidistance for dual prism setup for different central wavelengths. Prism angle β_p was fixed at 30°

Areas of nonequidistance less than 0.02% in the dual-prism setup are presented in Fig. 2. The figure is plotted for a pair of 30-degree prisms, β_0 is the angle of incidence on the first prism plane, β_1 is the angle between the prisms. The filled areas represent nonequidistance correction over 0.02%.

Although these areas do not overlap (as is seen in Fig. 2), the setup can be readjusted by only changing the tilt of the prisms, there is no need to create a new prism.

Acknowledgements

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OCT-STUDY OF NEONATAL SKIN STRUCTURAL FEATURES

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Abstract. The light-tissue interaction in the visible wavelength range is broadly used in infant intensive care, for instance, for transcutaneous bilirubinometry or oximetry. Information about optical properties of skin is very scanty, despite the fact that the majority of optical diagnostic methods in neonatology are immediately connected with the light-skin interaction. The optical properties of neonatal skin were studied by I.S. Saidi et.al [1] who measured the absorption and scattering coefficients as a function of age and found that the scattering coefficient increases with age as a result of skin maturation. The optical properties of neonatal skin as a function of age and phototype were investigated by a group of scientists from the Netherland Center for Biomedical Engineering and Physics [2]. The absorption (μ_a) and scattering (μ_s) coefficients in neonatal skin were measured in natural conditions at the wavelength between 450 and 600 nm. In the present manuscript we describe the first multifocal non-invasive morphological study that enabled us to define specific qualitative and quantitative features of newborns. The structure of neonatal skin in real conditions was studied by the method of optical coherence tomography (OCT) that allows noninvasive imaging of studied skin sections at a depth up to 1 mm in real time.

Ten newborn babies (2 girls and 8 boys) aging 1 to 28 days underwent OCT examination. Ten teenagers (4 girls and 6 boys) at the age from 12 to 17 years old were examined for comparison. The structural skin parameters were investigated at 14 points in different anatomic areas (hair-line, center of the forehead, cheek-bone, angle of eye, angle of mouth, shoulder, extensor and flexor surface of forearm, palm, umbilicus, jugular fossa, shin, foot rear, and heel). The teenagers and newborn infants were examined with approbation from the parents who signed written informed consent. The study was approved by the local ethic committee of the Federal State Institution Nizhny Novgorod Research Institute of Dermatovenereology (Protocol No.12 from 01.10.04). Morphological skin state was studied by an OCT device with a removable flexible probe with microscanner produced at the Institute of Applied Physics (IAP RAS), Nizhny Novgorod, with the following characteristics: radiation wavelength 920 nm, radiation source power at the probe output 1.5 mW, longitudinal resolution 20 μm , transverse resolution 25 μm , central wavelength 0.95 μm , scanning depth 1.5 mm, imaging time 1.5-2 s [5]. The OCT images of infant skin feature a strongly scattering surface layer of different height along the whole length varying from 14 to 42 μm in different localizations. In a reference group, this layer is either thinner, 7 to 14 μm or is poorly discernible. Reflection of probing radiation from the media interface contributes to formation of this layer (Fig. 1).

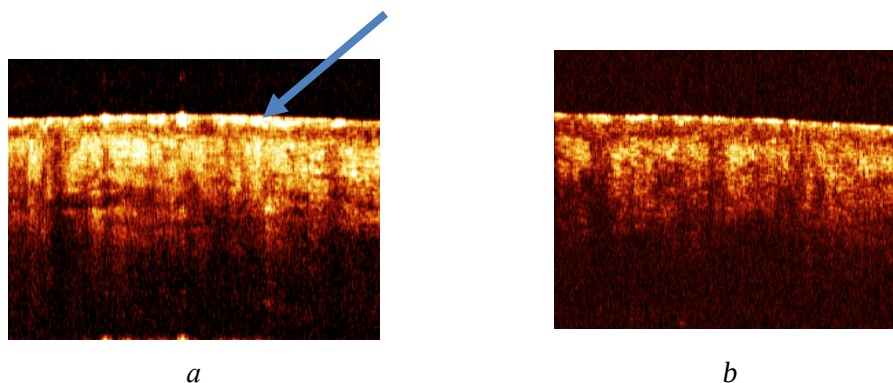


Fig. 1. Optical image of the face of a 13-days old newborn (*a*) and a teenager 14 years old (*b*)

Taking into consideration that the stratum corneum is an integral part of the epidermis and the total thickness of the infant epidermis is smaller than of the adult epidermis, we describe more qualitative than quantitative changes of the stratum corneum. In this case, the observable increase in the cellular layer fraction is the result of loose arrangement of the scales, their thickness and the air filling in the space between them.

The OCT images of palms and soles are also qualitatively different. For example, newborns have no typical pattern of thick skin with a pronounced stratum corneum and papillae. The optical image of thick skin in newborn infants has no typical layered structure, contrast and clear borders between the layers, which renders mathematical processing of images impossible (Fig. 2).

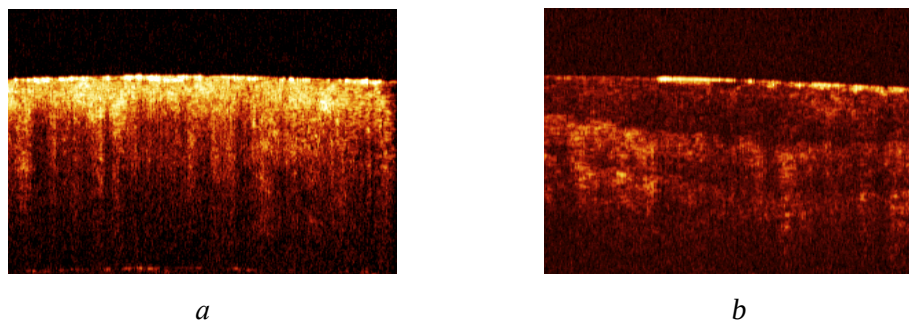


Fig. 2. Optical image and A-scan of thick palm skin of a newborn (a) and a teenager (b)

Investigation of structural skin parameters revealed that neonatal epidermis is thinner than teenage epidermis, with the value of parameters statistically less significant at the points of hair-line, angle of mouth, umbilicus, and foot rear. The minimal size of epidermis in both groups was recorded in the angle of eye.

The useful signal depth increased at all measured points of the neonatal skin images, with statistically significant differences in more than half the measurements.

Comparison of contrast in OCT images of neonatal and teenage skin did not reveal any regularity. The only fact worth mentioning is that in the area of the angle of eye, forearm, and leg contrast is statistically less significant in newborns.

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SIMULTANEOUS PHOTOACOUSTIC AND OPTICALLY MEDIATED ULTRASOUND MICROSCOPY FOR BIMODAL BIOIMAGING OF FUNCTION AND STRUCTURE

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Abstract. An experimental setup for combined Photoacoustic (PA) and optically mediated Ultrasound (US) microscopy is presented. A spherically focused 35-MHz PVDF ultrasonic detector with a numerical aperture of 0.28, a focal distance of 9 mm, and a bandwidth (-6dB level) of 24-MHz was used to obtain PA and US data by 3-mm imaging depth. A fiber-optic system was employed to deliver laser excitation pulses from a tunable laser to the studied medium. A single optical pulse was used to form both PA and US A-scans. The probing US pulses were generated thermoelastically due to absorption of backscattered laser radiation by the metallized surface of a PVDF film.

Photoacoustic (PA) imaging is a modern method of biomedical visualization based on detection of ultrasonic waves excited in the studied medium due to absorption of pulsed laser radiation by optical inhomogeneities [1].

The main advantage of PA imaging techniques compared to the purely optical methods is improved spatial resolution at depths from several millimeters to several centimeters. Modern pulsed lasers allow using wavelength tuning to achieve the maximum gradient of optical absorption of investigated structures as compared with the surrounding tissues. Therefore, it is possible to optimize PA contrast of endogenous light-absorbing agents (such as hemoglobin, melanin, water, etc.), which allows one to visualize the vascular pattern of tissues and to determine the oxygenation status of the local tissue. The use of exogenous contrast markers (organic dyes, nanoparticles, fluorescent proteins, reporter genes, etc.) enables PA molecular imaging [2] with enhanced contrast, which is unachievable by the conventional bioimaging techniques such as ultrasonography.

However, the combination of PA imaging techniques with standard active Ultrasound (US) methods appears in many cases quite justified [3] for the following reasons. First, standard ultrasonic detectors can be used for recording both US and PA pulses. Second, the US methods are aimed at imaging fundamentally different contrasts which are based on differences in acoustic impedances for various biological tissues. Therefore, extension of PA imaging to dual-modality PA/US imaging can provide complementary structural information about the tissues under investigation.

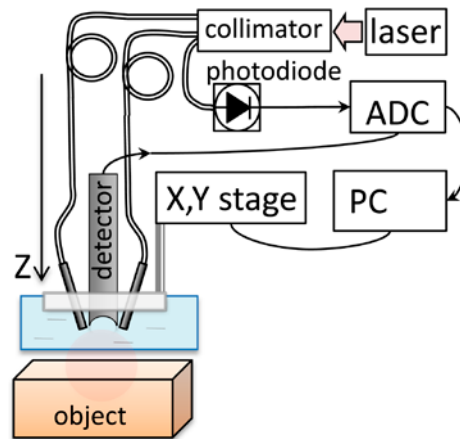


Fig. 1. The scheme of experimental setup for simultaneous US/PA imaging

In our work, we implemented an original system combining the US and PA microscopy techniques with excitation of ultrasonic pulses provided by the metallized surface [4] of a spherically focused ultrasound transducer (external electrode of a PVDF film) due to its absorption of pulsed laser radiation backscattered by the studied tissue. Using the same spherical surface both for generation and detection

of US pulses leads to effective multiplication of the detector's radiation pattern ensuring the improved spatial resolution. Also, our system does not require additional bulky elements intended for excitation of probing ultrasound pulses. Levels of acoustic signals recorded at the time of laser generation can be used in signal processing for normalization of US A-scans corresponding to different positions of the sensor over the medium surface. Therefore, excitation of external electrode of PA detector by back-scattered laser radiation can ensure a cost-effective upgrade from single-modality PA microscopy to dual-modality PA/US imaging.

The scanning head of the microscope (fig. 1) consists of a spherically focused PVDF detector which is surrounded by seven fiber bundles directed to the detector focal zone. The automation system allows the scanning head to move along the horizontal axes X and Y with the repetition rate of laser pulses (10 Hz) in an immersion chamber filled with distilled water. The stationary contact with the investigated object was ensured by the immersion chamber with translucent window at the bottom made of a polyethylene film.

The original signals from the sensor passed through the custom-made AD8099-based low-noise amplifier, the NI5761 14-bit 250-MS/s digitizer, and the Matlab built-in digital high-pass filter with a cut-off frequency of 1 MHz, which eliminated the low-frequency signal induced due to the absorption of the laser pulse by the detector surface). All 3D images were formed from the independent A-scans subjected to the Hilbert transform.

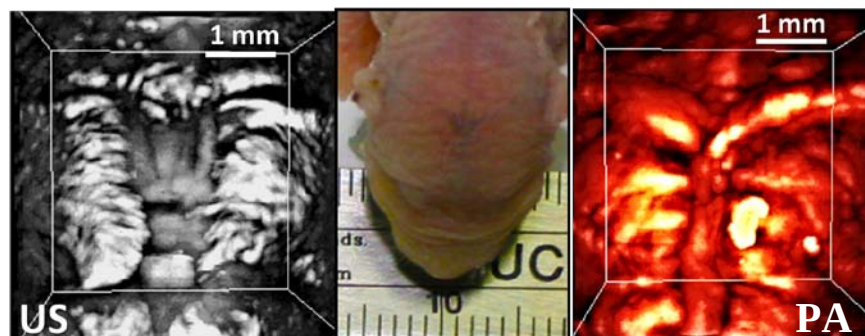


Fig. 2. Simultaneous US/PA images of rat brain *in vivo*

The results of two-dimensional imaging of rat brain *in vivo* by PA and US methods are presented in fig. 2. The acquisition time of a single 3D-scan consisting of 300 by 300 A-scans was limited by 150 minutes due to a laser pulse repetition rate of 10 Hz. For better visual contrast, signals with a level less than 25% off the maximum are shown black in fig. 2. A single laser pulse was used to form each A-scan. 584nm laser wavelength was used, which corresponds to identical absorption of Hb and HbO₂. The maximum pulse duration of this laser system was 18 ns.

Acknowledgements

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SHEDDING LIGHT ON RADIOTHERAPY: OPTICAL COHERENCE TOMOGRAPHY FOR ASSESSMENT OF RADIOBIOLOGICAL RESPONSES IN-VIVO

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Abstract. We report on optical coherence tomography research for examining X-ray irradiated normal and cancerous tissues. Both pre-clinical animal model studies of early effects, and a pilot human clinical study of long-term complications, are discussed. Textural analysis of OCT structural images and quantification of OCT microvascular images are presented, in an effort to develop quantitative image metrics that are sensitive to radiation effects. The radiation induced OCT signal changes, once quantified and validated, can help optimize and personalize radiation therapy, with ultimate clinical implementation made possible by the compact, practical and robust nature of OCT technology. Patient-specific individualized irradiations can then become feasible.

Introduction. Radiation therapy (RT) is a widely used cancer treatment modality (~50% of all cancer patients are treated with it), but its detailed mechanism of action and the variable patient responses are poorly understood. While DNA double strand breaks are the accepted cellular targets of radiation damage, the role of microvasculature, connective tissues, and the complex temporal interplay within these inter-related damage compartments are poorly understood, often leading to variable patient response (both in terms of disease control and in terms of complication rates). Thus, despite tremendous advances in imaging for RT (e.g., image-guided radiation therapy, IGRT), its use is primarily limited for treatment planning and geometrical targeting to ensure accurate dose delivery. The mechanism of variable tissue response remains unknown, and often several weeks or months are required before a post-treatment CT or MR reveals whether RT has been successful or not. This is not an ideal clinical situation.

We thus propose to use optical coherence tomography (OCT) to ‘shed additional light’ on radiotherapy. We examine both microstructural and microvascular OCT imaging modes, and develop various quantitative metrics to test their dependences on radiation dose. A pre-clinical study in immunocompromised tumour bearing mice concentrating on early (1-3 weeks) radiobiological response is presented. As well, we’ve recently initiated a pilot clinical study in head & neck cancer patients that experience late (6 months – 2 years) radiation toxicity in their oral mucosa. Initial results from this clinical pilot will be presented as well.

Results and Discussion. (1) *Early radiation effects, preclinical study with tumour-bearing nude mice in a dorsal window chamber model:* Both microstructural and microvascular OCT quantification studies were performed longitudinally for ~2 weeks following radiation dose delivery. The fluorescently-labeled ME-180 human cervical carcinoma cell line was used for tumour induction. Figure 1 shows speckle-variance (sv) OCT images of the microvasculature, and the resultant quantification of vascular pattern differences between normal and tumour regions. Similar analysis is currently underway to quantify radiation-induced effects (18 Grey dose levels).

Fig. 2 shows the results of OCT textural analysis, fitted with Gamma function often used in ultrasound for tissue characterization. Subtle but discernible trends attributable directly to RT dose deposition are seen.

Overall then, the preclinical OCT quantification of RT-induced microstructural and microvascular alterations is beginning to reveal some trends. Owing to the rather subtle nature of the radiobiological alterations and the inherent biological variability of *in-vivo* tissues, much more work is required to test the robustness of these initial metrics, examine their dependent on the RT dose levels, and test their utility in different model systems.

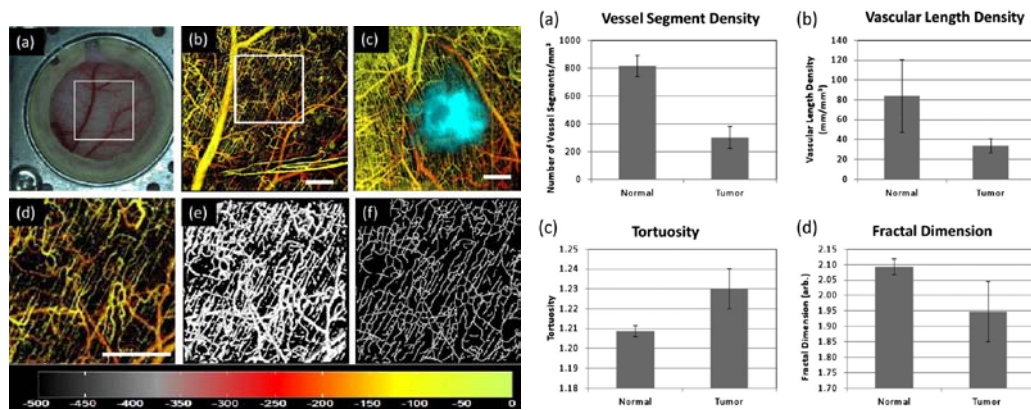


Fig. 1. Left panels – the svOCT images of the normal and tumour microvasculature in a mouse window chamber. The ME-180 tumour cell lines are fluorescent cyan. Colour bar at the bottom encodes depth, with yellow being the most superficial. Binarization and skeletonization are used for further analysis of the vasculature. Scale bars 1 mm. Right panels – the four resulting vascular metrics for normal and tumour vasculature. Similar quantification is underway to longitudinally monitor the resulting irradiation effects (from [1])

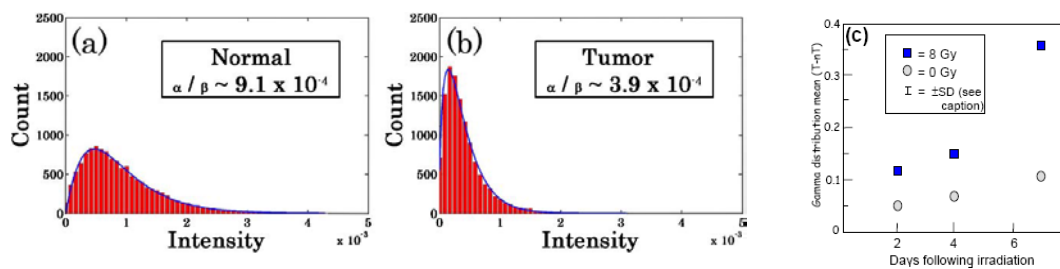


Fig. 2. Histograms of the non-log-compressed OCT image pixel intensity distributions in a 64×190 pixel (210 μm×630 μm) ROI region for (a) normal and (b) Me-180 tumor tissue 17 days after tumor implantation. The mean α/β parameter is from the Gamma function fit shown in blue (from [2]). (c) the ‘mean’ Gamma distribution parameter, plotted as a difference between tumour (T) and non-tumour (nT) regions for both irradiated and un-irradiated mice, at 3 times following 8 Gy dose. Significant changes following RT are seen, showing increasing differences with time

(2) Late radiation effects, clinical pilot study of head & neck cancer patients with oral mucosa radiation toxicity: We’ve recently got approval for a pilot study in 15 late radiation toxicity oral cancer patients and 6 healthy volunteers. We are part of the way thru this clinical pilot study, and present interim results below. Figure 3 summarizes the preliminary results of microvascular OCT analysis from 4 patients and 3 volunteers.

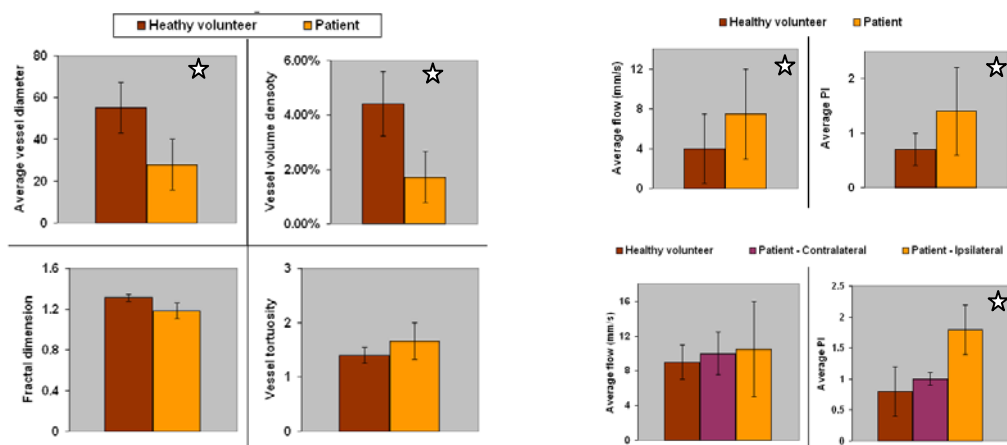


Fig. 3. Interim results from a pilot clinical study (7 subjects to date). Left panels – svOCT based microvascular metrics and quantification results. Right panels – analogous Doppler OCT analysis. ☆ = statistically significant differences between patients (n=4) and healthy volunteers (n=3); $p < 0.05$, paired T-test (after [3])

Conclusions. Novel platforms for acquisition and quantification of OCT microstructural and microvascular images was developed and used in *in vivo* animal and human imaging studies of early and late radiobiological effects, respectively. RT tissue effects are quite subtle and hard to discern, so advanced image analysis and signal processing are required. Nevertheless, preliminary OCT metrics of tissue radiation response are starting to emerge. Given the tremendous importance and prevalence of RT in combatting the cancer burden, these ‘shedding light on radiotherapy’ efforts will continue.

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NANO-SCALE CELLULAR IMAGING USING SUPER-RESOLUTION MICROSCOPY

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Abstract. Since the invention of the microscope, the spatial resolution that could be achieved with optical microscopes was limited by Abbe's diffraction limit. In the past decade, several techniques that improve the spatial resolution of optical microscopes beyond the diffraction limit have emerged, based on engineering of the point spread function, structured illumination, single molecule imaging, or fluctuation analysis. We present here the use of structured illumination microscopy for medical problems via morphological and functional analysis of cells and cellular structures.

Introduction

Optical microscopy (OM) is arguably the most important tool used to understand the structure and function of cells. OM is a mature technology that is able to non-destructively obtain 3D dynamic images of cells in real-time and is most often used with multi-colored fluorescent probes that selectively label structures or materials of interest. For these reasons, optical microscopy also continues to be an active and rapidly growing area of research, fueled by advances in optical technology, such as confocal optical microscopy, single molecule fluorescence detection, multiphoton excitation of fluorescence, and the continued development of novel optical probes, such as fluorescent proteins.

The principal drawback of optical microscopy is that subcellular objects and structures of interest that are much smaller than the illumination wavelength cannot be resolved (resolutions are approximately 250 nm laterally (xy) and 500 nm axially (z)), due to diffraction properties of light. Recently, several groups have addressed this shortcoming and developed techniques aimed at improving spatial resolution of optical microscopes [1-4]. The new techniques have lateral resolutions of 20–120 nm, which are well beyond the optical resolution achieved with conventional microscopes. Historically, the first super resolution microscopy technique that was developed

was Stimulated Emission Depletion microscopy (STED), which, by manipulating the beam profile of a 'quenching' laser, reduces the effective emission volume to achieve a lateral resolution of tens of nm [5]. Shortly afterward, photoactivation based techniques (including Stochastic Optical Reconstruction microscopy (STORM) [6] and Photoactivated Localization Microscopy (PALM) [7]) were invented, which take advantages of localizing photoswitchable

dyes (whose fluorescence status can be toggled with a UV laser beam) and by imaging them one-by-one to reach a lateral resolution as high as 20 nm. Meanwhile, Structured Illumination Microscopy (SIM) [8, 9] and some other techniques (such as super-resolution Optical Fluctuation Imaging (SOFI) [10]) were also developed. While the resolution of SIM is not the highest among super-resolution techniques, this method is fast (acquisition times are comparable to that of a confocal microscope), uses a relatively simple laser system, has no special requirements for sample staining (the sample just needs to be fluorescent and stable), offers resolution improvement in all 3 dimensions and can be capable of high penetration depth of imaging (up to approximately 30 microns) [3, 4].

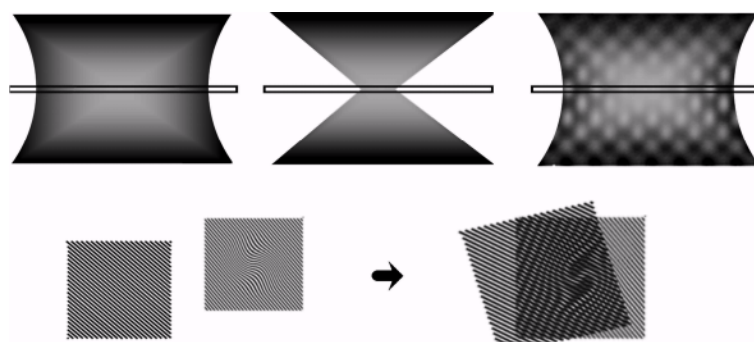


Fig. 1. Top: different illumination profiles near the sample plane in wide field (left), confocal, (middle), and structured illumination (right) configurations. Bottom: a moiré pattern generated from a high spatial frequency feature of the sample with a structured illumination pattern

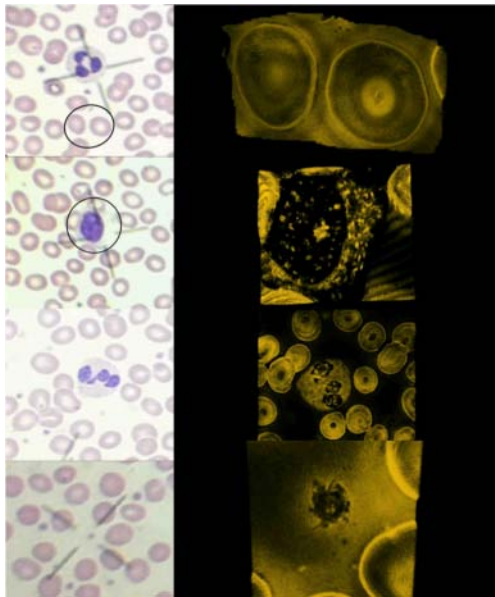


Fig. 2. Comparison of transmitted light (left column), and SIM (right column) images of red blood cells (top row), a monocyte cell (second row from the top), a neutrophil cell (third row from the top) and a platelet (bottom row). Note, the color in fluorescence images and in SIM images are pseudo-colors for rendering, not related to the color of the real fluorescence

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Resolution enhancement by SIM is determined by the spatial frequency of the illuminating pattern. This frequency is also limited by diffraction and typically results in a resolution enhancement of 2 (linear structured illumination) in each dimension, which corresponds to a factor of 8 improvement in the sample volume [1]. The resolution can be further enhanced if the fluorescent probes in the sample are made to produce a nonlinear response thus introducing higher-frequency harmonics into the effective illumination pattern (nonlinear structured illumination). One technique for obtaining nonlinear response from fluorescent probes is to increase the illumination intensity to the point where the emission rate starts to become saturated. This approach has recently achieved resolution enhancements of 5.5X [1] in which fluorescent beads with 50 nm diameter were fully resolved.

We will present here nanoscale imaging of cells using SIM. Live cells, as well as fixed pathology samples, will be visualized for structural and functional information that will be related to medical problems such as HIV infections or cytopathology of blood cells. As an example, in Fig.2 we present images showing detailed morphology of various types of blood cells, and platelets.

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FULL JONES MATRIX TOMOGRAPHIC IMAGING IN VIVO BY OPTICAL COHERENCE TOMOGRAPHY

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Abstract. The principle and application of Jones matrix tomography (JMT) is presented. JMT measures three-dimensional distribution of the Jones matrices and derives back-scattering intensity, Doppler shift, and phase retardation from the Jones matrix tomography.

Introduction

Optical coherence tomography (OCT) has become an integral tool of ophthalmology for a couple of decades. Although the structural investigation by OCT is important, the eye and eye diseases are associated with several other factors including microscopic tissue abnormality and abnormality in circulation.

In order to investigate these abnormalities, Doppler OCT [1] and polarization sensitive OCT (PS-OCT) have been widely studied [2, 3]. Doppler OCT obtains Doppler shift of the OCT probe beam by assessing the phase of OCT signals, and finally provides flow-specific contrast. On the other hand, PS-OCT is capable of providing birefringence tomography. As the birefringence of biological specimens is mainly generated by fibril extracellular materials, PS-OCT would provide additional contrast to OCT for discriminating several tissue types.

So far, Doppler OCT and PS-OCT have been regarded as two different extensions of OCT. However, by regarding the OCT as a tool to determine the comprehensive optical property of the sample, we can understand the relationship among these OCT extensions from a different perspective. Namely, to fully characterize the back-scattered probe beam from the specimen, three features of the light should be determined: intensity, phase and polarization. Similarly, to fully characterize the optical property of the tissue, we should determine its scattering intensity, phase shift, and polarization properties. Jones matrix measurement is one of the solutions for this characterization. From the Jones matrix back-scattering tomography (standard OCT), phase (Doppler) tomography, and birefringence tomography can be obtained.

In this talk, the newest version of our Jones matrix tomography [4] and its application to ophthalmology are described. The scattering, phase and polarization measurement are integrated into a single theory.

Jones Matrix OCT system

Our Jones matrix OCT is based on a swept-source OCT technology. The light source is a wavelength-scanning laser with a center wavelength of 1.06 μm , a scan range of 120 nm, and a -3-dB bandwidth of 100 nm (AXSUN Technologies Inc., NJ, USA). The scanning frequency of the light source is 100 kHz and it results in an A-line rate of the Jones matrix OCT of 100,000 A-lines/s. The probe arm consists of a delay-based polarization multiplexer. This multiplexer gives a different delay to horizontal and vertical polarization components of the incident probe beam. And hence two OCT signals corresponding to these two polarization states appear at different depths in the OCT image.

The OCT signals are detected by a polarization diversity detection scheme, the OCT signals correspond to a Jones vector at the detector. As shown in Fig. 1, the OCT images corresponding to each entry of the Jones vector are composed of two OCT cross-sections at two different depths. These images correspond to the two incident polarization states. Since each of the two detection channels provides two OCT signals, four OCT signals are acquired simultaneously.

These four OCT images correspond to four entries of a multiplication of Jones matrixes of illumination optics, round trip Jones matrix, and collection optics. The effect of illumination and collection optics is then cancelled by using the four OCT images at the surface of the sample, which are only affected by the illumination and collection optics. And finally, the back-scattering intensity tomography, i.e., standard OCT, the Doppler OCT, and phase retardation tomography are obtained from these four OCT images.

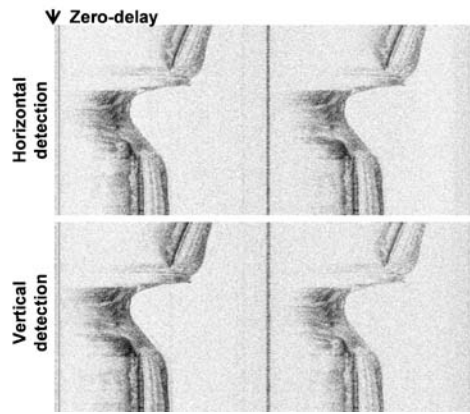


Fig. 1. Raw OCT images measured by Jones matrix. The upper image is of horizontal detector and the lower image is of vertical detector. Each image consists of two OCT images which correspond to the first and the second incident polarization states

***In vivo* measurement of human posterior eye**

Examples of *in vivo* polarization sensitive measurement of the human optic nerve head are shown in Fig. 2. The intensity image (a) shows well known tomographic structures of the optic nerve head including lamina cribrosa indicated by an arrow. The phase retardation (b) shows strong birefringence at the scleral canal rim which possesses high-dense collagen, and hence possesses high birefringence (circle). The en-face Doppler image (c) reveals the fine details of the vascular network.

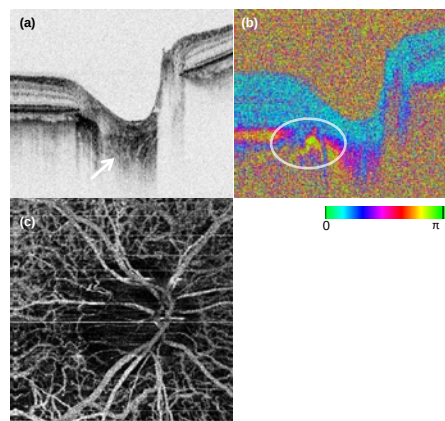


Fig. 2. Examples of tomography of an *in vivo* human optic nerve head. (a) Intensity images created as a maximum intensity composite of four entries of the Jones matrix, (b) phase-retardation image, and (c) en-face projection of the power of Doppler signal

Summary

This paper provided an overview of Jones matrix OCT including its principle and application to ophthalmology. Some more basic principles and clinical utilities will be discussed in the presentation.

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FLUORESCENCE IMAGING AND TOMOGRAPHY UTILIZING UPCONVERTING NANOPARTICLES AS CONTRAST AGENT

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Abstract. Upconverting nanoparticles (UCNPs) is a novel type of contrast agent for bioimaging. It possesses a number of very attractive properties, such as being excited at long wavelength in the tissue optical window, providing a background-free signal without any superimposed tissue autofluorescence and yielding an improved spatial resolution as compared to other fluorescence contrast agents. Despite the recent development in making the UCNPs much more efficient, a limited quantum yield is still an issue. We demonstrate how one can optimize the signal by employing pulsed excitation.

Summary

Imaging has become an increasingly important tool in drug discovery and development, as it provides a tool for minimally invasive monitoring. In vivo alterations at the organ, tissue, cell or even molecular level can be closely monitored in animal studies to improve the understanding of basic pathological physiology. Studies over days, weeks and months on each individual are possible with repeated imaging. With improved imaging capabilities it is possible to further increase the reliability and accuracy and thereby further facilitate translational research. All imaging modalities used clinically today are also available for preclinical imaging; while optical imaging is the most common modality used in small animal in vivo imaging due to its simplicity and cost-effectiveness. The main limitations of fluorescence small animal molecular imaging are tissue autofluorescence, poor resolution and poor light penetration, making it difficult to image deeply located regions with high sensitivity. Furthermore, photobleaching of the traditionally used fluorescence probes sets a limit for repeated imaging required in longitudinal studies. Upconverting nanoparticles (UCNPs) have a potential to overcome these limitations by their unique properties. UCNPs are synthesized as small crystals doped with certain trivalent lanthanide ions or transition metals. Common materials are ytterbium and yttrium in combination with small amounts of other ions, like for instance erbium (Er) or thulium (Tm). Such Yb/Tm-codoped UCNPs are excited at a wavelength of around 980 nm and emit light at 800 nm. Both the excitation and emission wavelengths are close to optimal for in vivo imaging in tissue. Due to the anti-Stokes shift of the upconverting signal, the signal can be truly background-free without influence from any tissue autofluorescence. This increases drastically the signal-to-background ratio, making it possible to measure very weak signals from tissue. Another benefit of UCNP imaging is the possibility to obtain an improved spatial resolution in the recorded images. This is due to the non-linear relation between the emitted signal and the excitation power. This non-linear dependence alters the sensitivity map for the nanoparticles within the tissue.

A main challenge with UCNPs imaging is the relatively low quantum yield. We have characterized particles designed for optimized quantum yield. We also show a significant improvement in spatial resolution for UCNPs as compared to conventional dye fluorophores, see Fig. 1. Then we also illustrate the use of highly efficient UCNPs for in vivo sentinel lymph node imaging in a rat. The results suggest that UCNPs can be very powerful as contrast agents for small animal optical imaging. Recent publications from the group on the topic can be found in Refs. [1-3].

Acknowledgements

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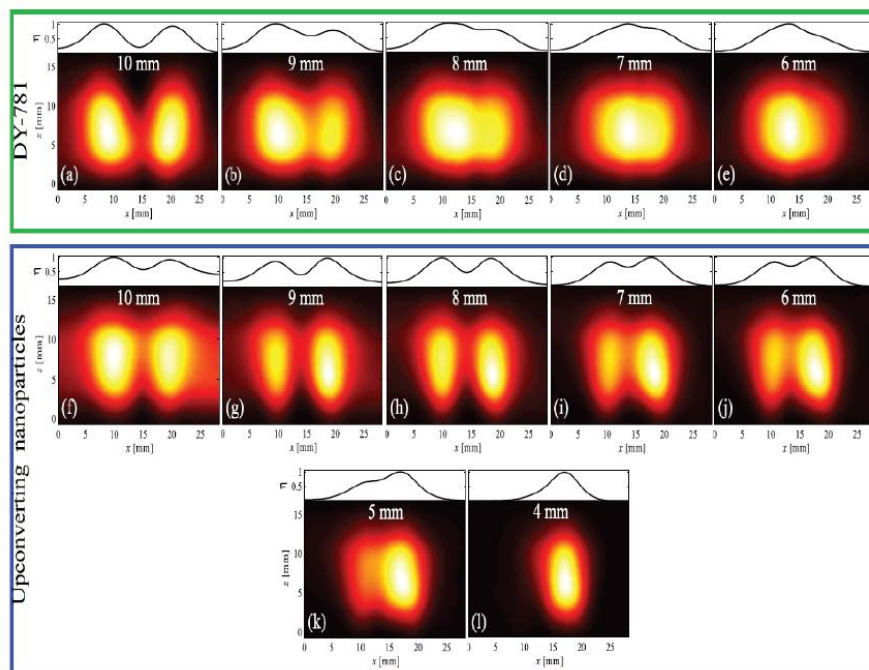


Fig. 1. Cross-sectional images of luminescence tomography reconstructions of a tissue phantom with two capillary tubes with varying separation distance and filled with the conventional DY-781 dye or with UCNPs. The use of UCNPs clearly leads to reconstructions with higher spatial resolution

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BAYESIAN TOTAL INTERNAL REFLECTION FLUORESCENCE CORRELATION SPECTROSCOPY REVEALS THE ORGANIZATION OF HIAPP-INDUCED DOMAINS ON PLASMA MEMBRANES

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Abstract. We extend our previously developed Bayesian analysis procedure for Fluorescence Correlation Spectroscopy (FCS) to enable its application to live-cell fluorescence imaging of Human Islet Amyloid Polypeptide (hIAPP) induced membrane domains. Results support the "carpet model" for the association mode of hIAPP-induced domains with the plasma membrane, consistent with previous observations in model membrane systems. The presented Bayesian approach provides an automated, unbiased procedure for multiple hypothesis testing of imaging-based FCS data, with broad applicability to resolving heterogeneous organization and dynamics of pathological biological membrane processes.

Introduction

FCS is a versatile tool for the measurement of molecular dynamics in living systems with single molecule sensitivity, including local concentrations, aggregation states, and transport mechanisms [1, 2]. The emergence of imaging FCS techniques including Total Internal Reflection FCS (TIR-FCS) [3] and Single-Plane Illumination-FCS (SPIM-FCS) [4] using cameras as detectors now enable parallel measurements at hundreds to thousands of spatial locations either on plasma membranes or within tissues with typically millisecond time resolution, making them ideal for resolving the spatial heterogeneity and dynamics of molecular processes in living systems. However, the large number of temporal autocorrelation functions (TACFs) with varying noise levels and unknown underlying physical processes that are generated by these camera-based techniques in a single acquisition require an automated, objective analysis procedure for their interpretation.

hIAPP has been suggested to interact with the cell membrane in forming aggregates and larger-scale fibrils that induce cytotoxicity that leads to β cell degeneration in the development of type II diabetes [5]. Recently, the Wohland group showed using TIR-FCS that hIAPP induced the formation of membrane domains containing dense lipids below the critical concentration for peptide aggregation [6]. However, the organization of these domains remains unclear based on conventional analysis performed using TIR-FCS. Here, we extend the Bayesian FCS analysis procedure recently proposed by the Bathe Group [7, 8] to TIR-FCS data and demonstrate its utility in resolving membrane heterogeneity in living cells treated with hIAPP.

Results and Discussion

We characterize the organization of hIAPP-induced domains forming on cellular plasma membranes using our previously published Bayesian procedure [7, 8]. A series of TIR-FCS measurements of the plasma membranes of SH-SY5Y cells stained with fluorescent probes DiI-C18 is recorded at five minute time intervals, with bright domains becoming clearly visible and increasing in size approximately 5 min after the addition of hIAPP. Compared to domains observed in phase-separated supported lipid bilayers (SLBs), conventional FCS analysis shows that domains in both systems exhibit low diffusivity inside the domains. However, the Bayesian model selection outcomes exhibit highly distinct patterns for these two systems. Two-component diffusion is detected mostly near the boundary of the domains for domains in SLBs (Fig. 1B, top), but is detected throughout the domains for hIAPP-induced domains (Fig. 1A, top row). The diffusivity distributions of the two components reveal a fast component D_{fast} coinciding with diffusion in the domain-free plasma membrane and a slow component D_{slow} , presumably consisting of diffusion of large peptide-lipid complexes (Fig. 1A, bottom row). The presence of two components inside the domain rather than one component suggests the observed domains are adsorbed on the surface of the membrane rather than embedded within it, resulting in TACFs containing contributions from both diffusion in the membrane and domains, which is further confirmed by simulations (data not shown).

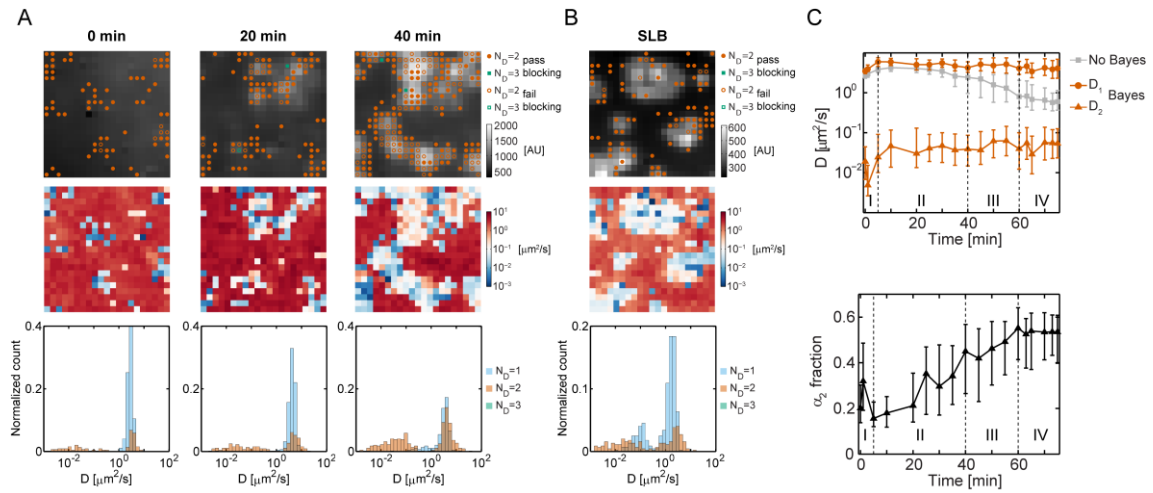


Fig. 1. Bayesian analysis supports the “carpet model” for organization of hIAPP-induced domains forming on plasma membranes. (A, B) (Top) TIRF images of DiI labeled (A) cell membrane at different time points and (B) two-component phase-separated SLB. Dots indicate pixels where multiple diffusing components ($N_D = 2, 3$) are detected. (Middle) Map of fit diffusion coefficients from preferred models. Diffusion coefficients of the slow component are shown for the two-component model. (Bottom) Distribution of fit diffusion coefficients from preferred models. (C) (Upper) Medians and quartiles of diffusion coefficients as a function of time from the conventional analysis (gray) and two-component regions in the Bayesian analysis (orange). (Lower) Slow component fraction in two-component regions as a function of time

Importantly, conventional analysis shows an initial increase in the overall D after addition of peptide, followed by a constant decrease until 60 min (Fig. 1C, upper panel). In contrast, Bayesian inference reveals an initial increase in both D_{fast} and D_{slow} in the two-component region. Subsequently, D_{fast} starts to decrease and recover slowly to its initial value, whereas D_{slow} remains the same within statistical error until the end of the measurement period. These results indicate association of monomeric hIAPP with plasma membranes increases membrane fluidity (D_{fast}), whereas the cell slowly recovers membrane fluidity to a normal level. The observed decrease in the overall D after 10 min is primarily due to the increasing domain area in the field of view as well as the increasing fraction of slow component α_{slow} (Fig. 1C, lower panel).

Conclusions

Our results suggest a domain formation model in which hIAPP forms a “carpet” on the plasma membrane that subsequently extracts lipids from the membrane by forming peptide-lipid complexes, consistent with a previous study using SLBs [6]. In contrast to that work, however, the present analysis reveals organization of membrane domains induced by hIAPP on plasma membranes and resolves multiple components in the complex overall diffusivity change, due to the novel inference ability of the Bayesian-FCS approach.

Acknowledgements

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USE OF GENETICALLY ENCODED SENSOR HYPER FOR STUDYING HYDROGEN PEROXIDE IMPLICATION IN THE MECHANISM OF CISPLATIN ACTION

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Abstract. Cisplatin is a cytotoxic agent causing breach of transcription and/or DNA replication. In addition to DNA damage cisplatin also induces the formation of reactive oxygen species (ROS), which leads to cell death. The role of ROS in the initiation of certain tumor cell death is not clear enough. For an assessment of a role of certain ROS in biological processes specific sensors are required. The goal of the current study was the investigation of the level of H₂O₂ in tumor cells in vitro via exposing cancer cells with cytotoxic drug cisplatin, using fluorescent genetically encoded sensor HyPer.

Materials and Methods

Cell line: human cervical adenocarcinoma HeLa Kyoto, transfected with the genetically-encoded cytosolic sensor for hydrogen peroxide HyPer [1, 2].

MTT assay

Assessment of cisplatin cytotoxicity in HeLa-Kyoto cell lines expressing the sensor HyPer has been performed using the MTT assay method. The test is based on the ability of mitochondrial dehydrogenases in viable cells to convert the water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan which crystallizes within a cell. [2]. Cisplatin concentrations ranged from 0 to 15 µg/ml.

There were two types of the MTT assay. In the first one, the cell viability was determined in 24 hours after cisplatin addition, in the second one in 2 hours. The absorbance of the resulting solution in DMSO formazan was measured on a spectrophotometer tablet Synergy MX (BioTek, USA) at a wavelength of 570 nm. The color of the cells not treated with cisplatin was taken as a 100% survival rate. Graphs of percentage cell survival depending on cisplatin concentration were averaged according to four experiments in each case. The results of MTT assay curves were constructed of cell viability HeLa-Kyoto-HyPer-cyto from cisplatin concentration in the medium after 24 hours (Fig. 1 A) and 2 hours (Fig. 1 B) incubation. Data are presented as means and standard deviations.

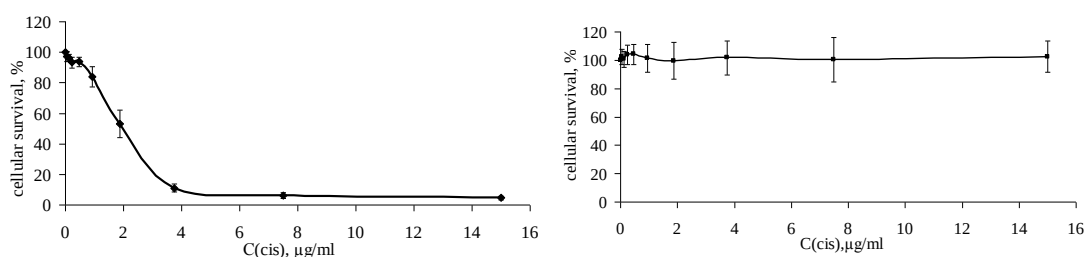


Fig. 1. Effect of Cisplatin on cell viability HeLa-Kyoto-HyPer-cyto.

A – 24 hours incubation with drug. B – 2 hours incubation with drug

Two drug concentrations: 3.85 µg/ml that causes 90% cell death and 1.9 µg/ml that causes 50% cell death (IC₉₀ and IC₅₀ respectively) were chosen for the further study of H₂O₂ dynamics. MTT test incubation with cisplatin for two hours showed no change in cell viability in culture regardless of the drug concentration.

Laser Scanning Microscopy

The dynamics of intracellular H₂O₂ concentration has been estimated using Laser Scanning Microscopy. The fluorescence of cells was observed by the laser scanning microscopy system LSM

510 Meta based on inverted microscope Axiovert 200 M (Carl Zeiss GmbH, Jena, Germany). The fluorescence was excited by the argon laser sequentially at two wavelengths: 458 nm and 488 nm. The registration of fluorescence was carried out using oil immersion objective 40h/1.3 in the range of 500-530 nm. Changes of the level of hydrogen peroxide were determined by the change in the ratio of fluorescence signals (F_{488}/F_{458}) from the cells at appropriate excitation wavelengths.

Cisplatin solution was added directly to the medium into a Petri dish. Fluorescent images were recorded every 60 seconds for 30 minutes. According to the ratiometric fluorescence monitoring, the addition of the indicated concentrations of cisplatin induced a transient increase in the level of H_2O_2 in the cell line HeLa-Kyoto-HyPer-cyto (Fig. 2). Figure 2 shows a F_{488}/F_{458} ratio level according to the averaged data on the level of fluorescence obtained from two (IC50) or three (IC90, control) 7-17 independent experiments for each cell. Data are presented as mean and error of mean.

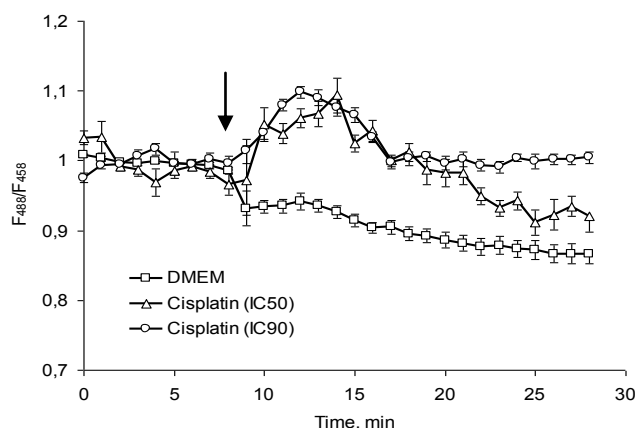


Fig. 2. Dynamics of F_{488}/F_{458} cells in response to the addition of cisplatin (IC50, IC90) and medium without cisplatin (marked by an arrow during the addition of the drug or the environment)

Immediately after the addition of the drug in the incubation medium an increase of the F_{488}/F_{458} ratio was observed, that lasted for about 5 minutes. Further, 10 minutes after cisplatin addition, the concentration of H_2O_2 fell again, as was evidenced by the reduction in F_{488}/F_{458} ratio to reference values or below them.

Discussion

In our work we investigated the dynamics of the hydrogen peroxide level in tumor cells under the influence of a cytotoxic agent cisplatin and demonstrated its transient increase immediately after the exposure. It was shown that the amplitude and duration of the “surge” does not depend on the concentration of the drug. Results of “two-hours” MTT-test confirmed the absence of loss of tumor cell viability for this period. Therefore, this increase cannot be explained by the release of the peroxide in the process of cell death. Taking into account HyPer sensitivity to pH changes, it is necessary to exclude its influence on cisplatin-induced cell answer in the further research.

Acknowledgements

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SOME STRATEGIES TO MEASURE INTRACELLULAR SODIUM CONCENTRATIONS

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Abstract. Fluorescent ion indicators provide the possibility to measure ion concentrations non-invasively in living tissues. Although many indicators are available for multivalent cations, only few indicators allow for measurements of physiologically important monovalent cations, such as sodium. Reliable ratiometric sodium indicators that can be excited at visible wavelengths are presently not available. In this study, we discuss several strategies to overcome this gap, including fluorescence lifetime measurements of non-ratiometric indicators such as Sodium Green, synthesis of ratiometric nanosensors, and synthesis of new fluoroionophores.

Introduction

In cell physiology sodium plays a crucial role. Mammalian cells maintain a large electrochemical gradient for sodium ions across the plasma membrane. This gradient provides the basis for rapid electrical signaling in many excitable cells. It also supplies the energy for important secondary active transport processes, in which the sodium influx along the gradient is used to power the co- or counter-transport of other ions or nutrients. For an understanding of the molecular action of these membrane proteins, and their role under physiological and pathological processes, it is necessary to measure the extra- and intracellular sodium concentration.

This task can be ideally fulfilled by fluorescent indicators that bind selectively sodium ions and change their absorption and/or emission properties upon binding. However, despite considerable efforts in synthesizing new compounds, only a few indicators, such as Sodium Green, have been proven to be suitable for measurements in aqueous solutions and biological specimens [1, 2] Sodium Green has the advantage that it can be excited in the visible range. Since its emission wavelengths are well separated from cellular autofluorescence, it can also be used under conditions where cellular autofluorescence is likely to change.

Fluorescence intensity and fluorescence lifetime measurements of Sodium Green

Upon binding to sodium ions, Sodium Green exhibits an increase in fluorescence intensity with little shift towards longer wavelengths (Fig. 1B). Figure 1 C shows the relative fluorescence ($F/F_{\min}$) as a function of $[Na^+]$. The fit of the data yields a value of 8.6 mM for Sodium Green's dissociation constant (K_d). In solutions containing both sodium and potassium the K_d is slightly elevated to a value of 22.4 mM (Fig. 1C). By knowing these data it is, in principle, possible to determine $[Na^+]$ in a biological sample.

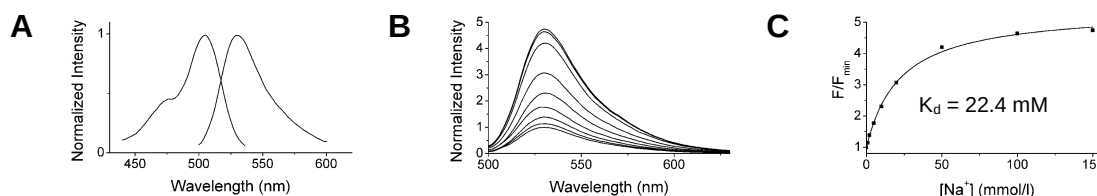


Fig. 1. Optical and chemical properties of free Sodium Green. **(A)** Fluorescence excitation ($\lambda_{em} = 530$ nm) and fluorescence emission spectrum ($\lambda_{ex} = 505$ nm) of Sodium Green in buffered solution (pH 7.4) containing 0 mM sodium. **(B)** Fluorescence emission spectra of Sodium Green ($\lambda_{ex} = 488$ nm) at sodium chloride concentrations of 0 (bottom), 1, 2, 5, 10, 20, 50, 100 and 150 mM (top). For all solutions containing less than 145 mM sodium chloride, potassium chloride was added so that the total cation concentration was 145 mM. **(C)** Fluorescence intensity as a function of free sodium concentration. The fit yielded a K_d of 22.4 mM

However, as Sodium Green is not a ratiometric dye there is no way to correct for inhomogeneities in the distribution of the indicator. High fluorescence signals may not only be due to a high sodium concentration, but can be caused by dye accumulation in parts of the cell. Likewise inhomogeneities in the illumination path can bias the fluorescence signal.

Fluorescence lifetimes are independent of these factors. They are a property of the fluorophore and its environment. Since sodium binds to Sodium Green, one should expect that it has some effect on the excited state lifetime. If one fluorescence lifetime component can be attributed to the sodium-free form of the dye, and one component to the sodium-bound form, then the overall fluorescence decay should exhibit a biexponential time course. The fit with a biexponential model, however, does not yield acceptable results. The fitted curves deviate considerably from the measured intensity decays, and the residuals are not randomly distributed [3-6] Apparently, the fluorescence decay cannot be explained by such a simple model, and most likely both forms, the sodium-free and the sodium-bound form, exist in several conformational states, with different lifetimes contributing to the overall fluorescence decay.

Sodium nanosensors

In addition to this, both, intensity and fluorescence lifetime measurements are biased by interactions of the dye with intracellular components, which make the analysis of the fluorescence data even more difficult. Figure 2A demonstrates the effect of proteins on the fluorescence properties of Sodium Green. In aqueous solution of 5 % bovine serum albumin (BSA) the emission intensity of Sodium Green is considerably higher than in a protein-free solution with the same $[Na^+]$. If the protein composition and concentration is unknown or even varies during an experiment, a reliable measurement of $[Na^+]$ is almost impossible.

These disadvantages can be overcome by incorporating the dye into a nanoparticle structure which is permeable to the analyte, but not to proteins (Fig. 2B). Figure 2C shows that Sodium Green incorporated in the polymer matrix of such nanoparticles retained its ability to respond to changes of $[Na^+]$ whereas it has become insensitive to the influence of BSA. Another advantage of this approach is that a second dye can be readily incorporated in the polymer matrix, whose fluorescence signal can then serve as a reference to indicate the nanosensor concentration.

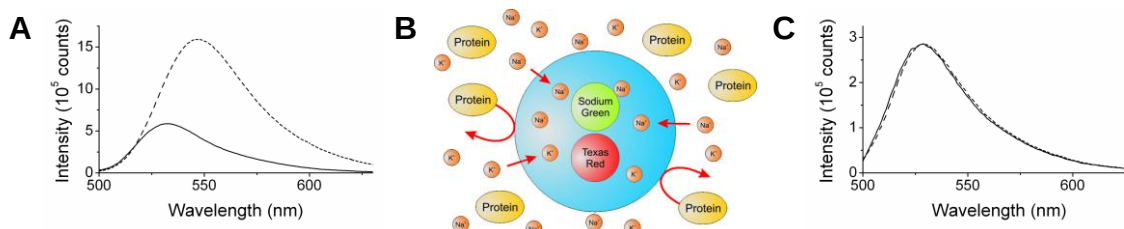


Fig. 2. Effect of BSA on dissolved Sodium Green and Sodium Green embedded in polyacrylamide nanoparticles. **(A)** Fluorescence emission spectrum of free Sodium Green in the absence (solid line) and in the presence (dashed line) of 5 % (w/v) bovine serum albumin (BSA). Spectra are normalized to the peak of the fluorescence spectrum in the absence of BSA. **(B)** Schematic of the sodium nanosensors. **(C)** Fluorescence emission spectrum of Sodium Green incorporated in polyacrylamide nanobeads in the absence (solid line) and in the presence (dashed line) of BSA. The incorporated indicator is insensitive to the addition of BSA: Both, intensity and peak wavelength do not change in the presence of BSA

Acknowledgements

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NANODIAMOND-HEMOGLOBIN COMPLEX DESIGNED FOR ARTIFICIAL BLOOD SUBSTITUTE

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Abstract. Nanodiamond (ND) has been proven as a convenient platform for bio/medical imaging and delivery of drugs and other substances. In this work we discuss how diamond nanoparticles can be used for delivery of oxygen at ND circulation in the blood and how ND can be used for development of nanoparticles-based blood substitutes. To study the interaction of diamond nanoparticles with hemoglobin (Hb) and with plasma proteins (albumin), the proteins were adsorbed on ND; the ND-Hb or ND-albumin-Hb complexes were characterized using spectroscopic methods (Raman, fluorescence, FTIR spectroscopy). The effect of the ND complexes on blood components and surrounding tissues at circulation in the blood is discussed.

Recently, nanodiamond (ND) is considered as an effective base for a number of bio applications [1]. ND's spectroscopic (Raman and fluorescence) and surface properties, chemical stability and biocompatibility add on the feasibilities for these purposes. Examples of ND's bio applications and its non-cytotoxicity have been successfully demonstrated for different cancer and normal cell lines. However, few publications exist concerning ND interaction and applications on tissues/organs levels; and the ND effects on the higher levels of biological system organization are important for use in biomedical researches and in medicine. One of the most critical peculiarities is its interaction with blood, such as integrative tissue. In this presentation the interaction of ND with blood (cells) and blood components is studied with aimed at estimating the ND biocompatibility with blood and understanding the possibility to use ND for delivery of oxygen/for development of nanoparticles-based blood substitutes.

Spectroscopic properties of ND are used for the study of the interaction and applied for delivery tracing and visualization. Previously, confocal microscopy, Raman spectroscopy and UV-visible absorption are used to characterize the interaction with red blood cell (RBC) membrane and the effect of ND on the oxygenation and deoxygenation processes of RBC. The effect of ND on the blood rheology is also discussed with analysis of RBC aggregation and deformability [2]. The ND interaction with blood plasma and blood plasma components, particularly, with the proteins (albumin, immunoglobulins) adsorption, their structure and functions [3] and ND effect on blood clotting factor are also studied. The predominant component of RBC is Hb, with its main physiological function to transport oxygen from the lung to the tissue cells and *back* transport of carbon dioxide. We study the interaction of ND with Hb, Hb adsorption, the effect of ND on the Hb oxygenation and deoxygenation for (1) understanding of the ND effect on blood; (2) because Hb is considered as a promising candidate for development of Hb-based blood substitutes (hemoglobin-based oxygen carriers, HBOC).

The need in artificial blood is determined by problems of medicine/physiology, medicine ethic and safety. Recently, two main types of blood substitutes are under development – HBOC and synthetic substitutes [4]. Purified cell-free Hb derived from both human and animal blood has very different properties from Hb contained within RBCs, including oxygen affinity and toxicity. To solve these problems cellular oxygen carriers were developed based on Hb encapsulated in vesicles/liposomes using biodegradable polymers or lipids but their use is limited first of all by their physical and chemical stability at circulation in blood and in tissues. To increase the stability and circulation time it is proposed to use nanoparticles as artificial oxygen carriers for Hb-based cellular-like systems [5]. In the presented work we discuss ND using for that purpose: surface properties of ND that allow constructing ND conjugates with molecules of interests; we have already shown that ND of proper properties and concentrations does not affect the process of RBC oxygenation/deoxygenation and does not disturb the blood rheology properties and can co-exist and co-function with red blood cells without destroying RBC's oxygen-carrying capability [2]; ND can be bi-functionalized via orthogonal coupled linker by two different molecules (e.g. Hb and some drug) [6], as well as ND allows tracing its circulation using spectroscopic properties [1].

The complex of Hb with ND was obtained by Hb adsorption on ND of sizes 50-500 nm. The complex properties and it's applicability as oxygen-carrier were analyzed using spectroscopic

methods, dynamic light scattering and surface charge measurement. Fluorescence imaging of 500 nm ND-Hb forming complex was observed (Figure 1). The ND fluorescence originated from ND N-V⁻ defects, centered at 690 nm is observed. Hb dyed with DIOC5 was excited at 488 nm and emission was collected in the 500-510 nm range. Oxygenation and deoxygenation of Hb in ND-Hb was observed using UV-visible absorption spectroscopy and Raman spectroscopy according to the method from [2].

To improve the complex stability and Hb functionality, complexes of Hb-albumin-ND have been prepared and analyzed. It has been shown that albumin can assist to support normal functional state of adsorbed Hb [7]. We have observed that serum albumin layer on the ND surface can prevent aggregation of ND, promote further adsorption of Hb the process of oxygenation and deoxygenation of Hb, improve the stability of the complex.

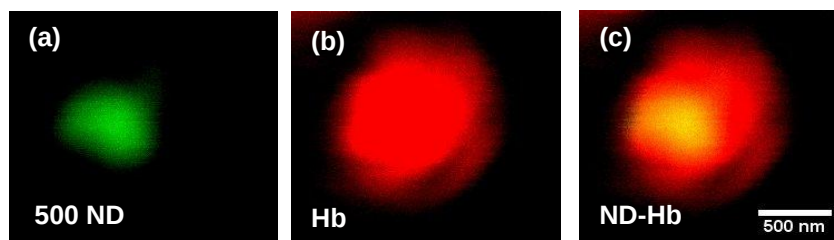


Fig. 1. Fluorescence imaging of ND-Hb complex. The ND fluorescence was excited at the wavelength of 633 nm; the fluorescence originated from ND N-V⁻ defects centered at 690 nm was observed. The fluorescence from Hb dyed with DIOC5 was excited at 488 nm and emission was collected at 500-510 nm

In conclusion, ND biocompatibility, possibility to conjugate (in this case to adsorb) biologically active molecules and to vary these molecules properties and functions make ND promising not only for drug delivery, but also for development of blood substitutes or multifunctional complex with oxygen-carrying properties.

Acknowledgements

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IMAGING OF CLEARED BIOLOGICAL SAMPLES WITH THE ULTRAMICROSCOPE

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Abstract. We report on new developments in Ultramicroscopy to improve imaging of large cleared biological samples. Objective devices that allow high resolution imaging through 10 mm of clearing solutions are described as well as special optics to create extra thin light sheets.

In the last years we have developed a special Ultramicroscope (light-sheet microscope) for visualizing neuronal networks in whole brains. In the Ultramicroscope whole cleared brains are illuminated with a sheet of light and the optical sections are used for 3D reconstructions. This approach allows one to employ also low power, wide field objectives for imaging of large samples.

By clearing neuronal tissue with organic solvents (BABB) after dehydration, we could visualize GFP-labelled neuronal networks in the whole brain [1]. Improving our clearing technology by using tetrahydrofuran for dehydration and dibenzylether (THF/DBE) for clearing we were able to image GFP-labelled axons even in heavily myelinated spinal cord [2, 3]. Also nervous and muscle structures in *Drosophila melanogaster* can be imaged [4]. Our and other clearing solutions have non standard refractive indices. Due to a heavy refractive index mismatch imaging in these solutions with e.g. air or water immersion objectives gives therefore suboptimal results. We thus developed special objective devices that allow refractive index matched imaging. We show that high resolution imaging through 10 mm clearing medium is possible (Fig. 1).



Fig. 1. *Drosophila melanogaster* imaged through 10 mm of clearing solution with the Ultramicroscope

Furthermore we substantially increased the axial resolution of our light-sheet microscope by developing completely new optics for light sheet generation. These optics create an extremely thin light sheet by the use of a Powell- and several aspheric lenses. As light sheet thickness determines the axial resolution it is of pivotal importance for the performance of the light-sheet microscope. Our light sheet is static and will thus in future allow combination with other microscopic techniques which need constant nonscanned illumination. Examples for the application of the ultramicroscope are given.

Acknowledgement

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REAL TIME MICROENDOSCOPY

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Abstract. A technical feasibility of autofluorescence microendoscopy in breast interstitially has been assessed as successful. Malignant tumor can be clearly identified. We also present the operation principle as well as the preliminary experimental results of a new type of micro size multicore fiber that enables imaging through blood vessel phantoms. Imaging of a manipulated micro wire through a drilled phantom is presented.

Keywords: Cancer margin delineation, endoscopy, microendoscopy, autofluorescence imaging, surgical guidance, multicore fibers

A technical feasibility of interstitial autofluorescence microendoscopy in breast as well as in blood vessels phantoms has been assessed as successful. Malignant tumor can be clearly identified. We assessed whether there are distinct changes in the tissue autofluorescence images between malignant and benign tissues that potentially can facilitate visualization of lesions that are not seen under conventional white light ductoscopy via breast ducts [1] and interstitially (Figure 1 a-b).

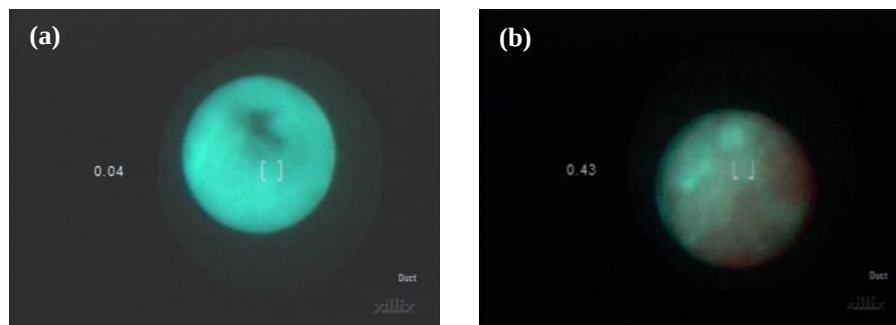


Fig. 1. Interstitial autofluorescence microendoscopy: Normal region (a) and Ductal carcinoma (b) occupying the whole field of view can be recognized as reddish patches. The forward-looking field-of-view view is approximately 1 mm. The microendoscope had 0.7 mm diameter and 3000 pixel resolution

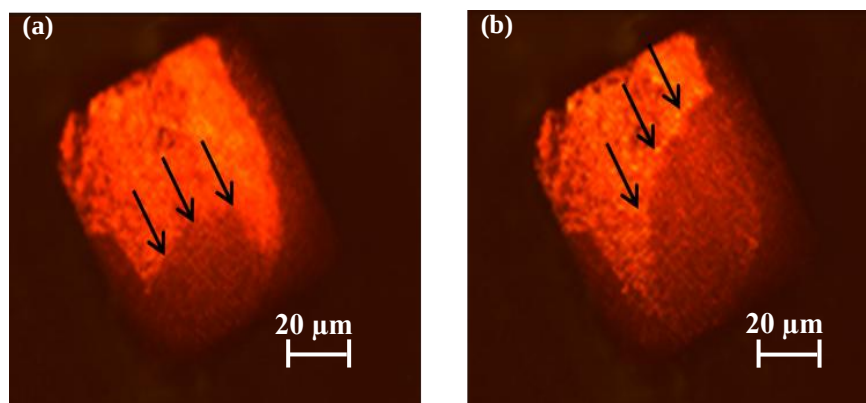


Fig. 2. Imaging of a manipulated micro wire (indicated by the solid arrows) by the multicore fiber (200 micron diameter, 5000 pixels) inside a hemoglobin mixture. (a) and (b) are the micro wire imaged at different orientation and positions

We also present a new type of a flexible micro probe consisting of multiple core fiber that may facilitate high resolution imaging capabilities as a microendoscope. The main advantages of the suggested micro probe are related to the fact that its external diameter can be relatively small (100-200 μm) although providing high resolution imaging up to 1,250-5,000 pixels. Applying a super-resolution technology [2], the resolution can be further enhanced by at least an order of magnitude. Such a micro probe can be also used for interstitial examinations or in blood vessels (Figure 2 a-b).

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MEASURING OF pH IN TUMOR XENOGRAPHS USING NEW GENETICALLY ENCODED SENSOR

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Abstract. Measuring of pH in tumor is an important task for its successful treatment. This work is aimed at the development of the method of pH registration in the animal tumor models. The study was performed using nude mice with tumor expressing genetically encoded sensor for pH. Measuring of pH was based on a ratio of fluorescence intensity at two different wavelengths. The data on fluorescence whole-body imaging were obtained by IVIS Spectrum system and confirmed by morphological investigation of the tissue sections. The possibility of using the unique genetically encoded sensor for ratiometric pH imaging in the tumors *in vivo* and *ex vivo* was shown for the first time.

THE INTRACELLULAR DISTRIBUTION OF GOLD NANOPARTICLES STABILIZED BY VARIOUS AGENTS

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Abstract. The aim of our study was an integrated investigation of gold nanoparticles penetration into cancer cells. SKOV-3 line cells were incubated with gold nanorods stabilized with Pluronic F127, chitosan and polyethylene glycol with molecular weight 6000 Da and 40000 Da. Gold nanoparticles–cells interaction was studied by transmission electron microscopic and two photon luminescence microscopic techniques. It was found that nanoparticles stabilized with Pluronic F127 and chitosan penetrated into cytoplasm and karyoplasm. PEG-stabilized nanoparticles were located outside of the plasma membrane mainly.

Gold nanoparticles are promising agents for various biomedical applications owing to their unique physical and chemical properties [1]. For example, they can be used for drug delivery, including anticancer drugs [2]. In addition, gold nanoparticles can be employed as contrast agents for optical imaging and as agents for tumor treatment [3]. However, mechanisms and features of gold nanoparticles–cells interaction remain unclear.

Therefore, the research is devoted to an integrated study of the interaction between cancer cells and gold nanoparticles stabilized with various agents by high-resolution microscopy techniques.

The work was carried out on human ovarian adenocarcinoma SKOV-3 cells. The gold nanorods used in this work had a plasmon resonance peak at 800 nm. The nanoparticles were stabilized with Pluronic F127, chitosan or polyethylene glycol with 6000 Da and 40000 Da molecular weight. Gold nanoparticles penetration and intracellular distribution was investigated by the transmission electron microscopy (TEM) and two-photon luminescence microscopy (TPL) techniques. Nonlinear luminescence was excited by 800 nm femtosecond laser and detected in the 565-615 nm range.

Preliminarily SKOV-3 cells without nanoparticles were examined by TPL to detect autofluorescence in the used spectral range. It was found that the cells had low level of autofluorescence, so gold nanoparticles could be easily detected in the cells.

After 1.5 h incubation a few agglomerates of the gold nanoparticles stabilized by Pluronic F127 were detected on the cells membrane and in the cytoplasm. Fluorescence signal intensity increased 25-fold as compared with the control. TEM confirmed that gold nanoparticles penetrated into cytoplasm. Single nanoparticles were found in mitochondrion. It should be noted that a few nanoparticles agglomerates were detected in cell nucleus. All found nanoparticles had a size of 60-150 nm which suggested their aggregation. After 3 h incubation the quantity of the gold nanoparticles penetrated into nucleus increased, and the nanoparticles were located in cytoplasm and mitochondrion. After 6 h incubation fluorescence signal intensity increased 39 times as compared with the control (fig. 1 a).

Gold nanoparticles stabilized with chitosan were practically absent in cells after 1.5 h incubation. Single gold nanoparticles agglomerates were detected on the membrane and in the cytoplasm of some cells. Fluorescence signal intensity was five times higher as compared with the control. These results were confirmed by TEM. The nanoparticles had the size of 110-240 nm. After 3 h incubation the quantity of the gold nanoparticles penetrated into cells increased insignificantly. Gold nanoparticles agglomerates were found in the cytoplasm and karyoplasm. Fluorescence signal intensity increased 15 times as compared with the control. Single nanoparticles were found in mitochondrion (fig. 1 b).

It was found that the gold nanoparticles stabilized with PEG 6000 Da were localized outside of cells membrane after 1.5 h incubation. Fluorescence signal intensity was 26 times above the control. Single gold nanoparticles agglomerates were detected in the cytoplasm. After 6 h fluorescence signal intensity increased 100 times as compared with the control, but gold nanoparticle quantity in the cytoplasm was stable (fig. 1 c).

The same tendency was shown for nanoparticles stabilized with PEG 40000 Da. The gold nanoparticles agglomerates were seen outside of the cells membrane and did not penetrate into the cells. Fluorescence signal intensity increased 16 and 132 times in comparison with the control after 1.5 and 6 h incubation correspondingly (fig. 1 d).

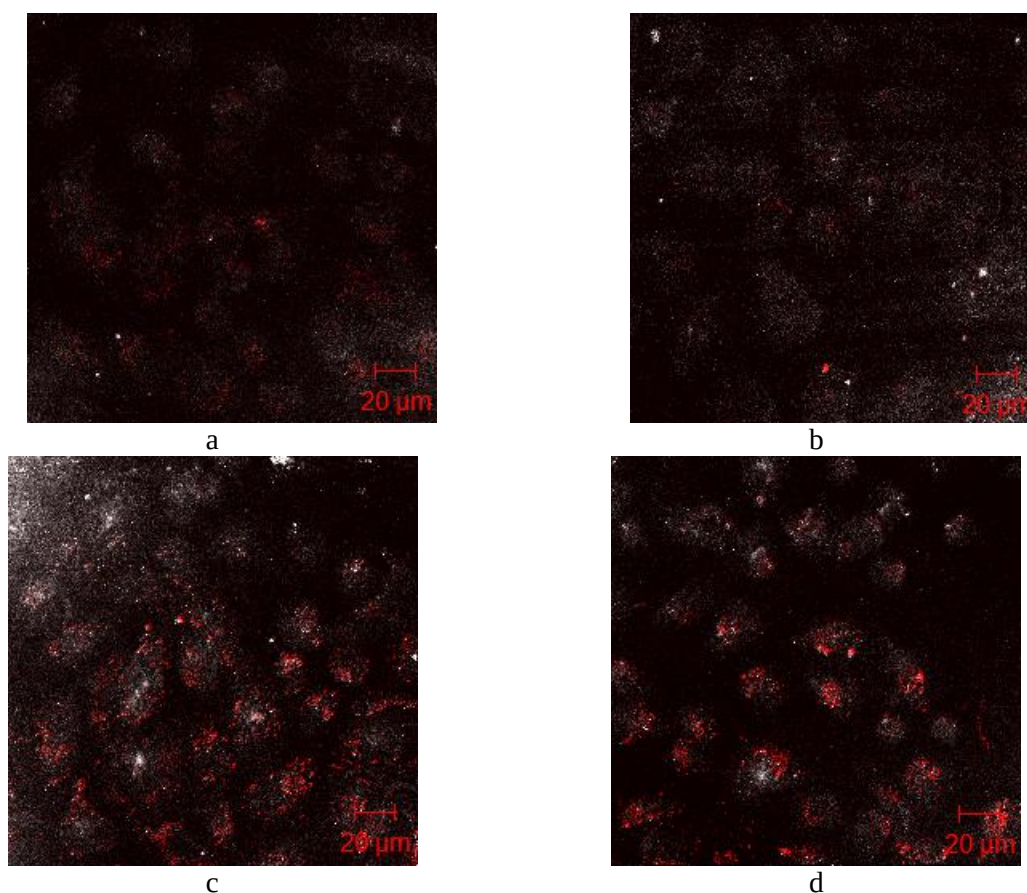


Fig. 1. TPL images cells contained gold nanoparticles stabilized by different agents after 6 h incubation:
a – pluronic F127, b – chitosan, c – PEG 6000 Da, d – PEG 40000 Da

In summary, we have shown that the gold nanoparticles stabilized with Pluronic F127, chitosan or PEG 6000 Da and 40000 Da can penetrate into cells, but the dynamics is different. The gold nanoparticles stabilized with Pluronic F127 penetrated into cells within the first hour and then their quantity increased. Chitosan stabilized gold nanoparticles penetrated slower and in less quantity than Pluronic stabilized nanoparticles. Gold nanoparticles stabilized by PEG 6000 Da and 40000 Da mainly located outside of the plasma membrane.

Acknowledgements

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CHLORIN e_6 FUSED WITH A COBALT-BIS(DICARBOLLIDE) NANOPARTICLE: SCYLLA AND CHARYBDIS FOR CANCER CELLS

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Abstract. Conjugate of chlorin e_6 derivative with cobalt-bis(dicarbollide) nanoparticle was created that fluoresces in a red region (670 nm), accumulates readily in cancer cells (ratio of cytoplasmic to extracellular concentration of 50 to 80), delivers more than 10^9 boron atoms per cell and possesses high phototoxicity for cancer cells ($LD_{50} \sim 80$ nm). It is nontoxic for cells without activating stimulus (photons, neutrons). Nanoconjugate properties are suitable to destroy cancer cells with boron neutron capture therapy or photodynamic therapy as well as to perform fluorescent tumor diagnosis.

Boron-neutron capture therapy (BNCT) and photodynamic therapy (PDT) are potent approaches to cancer treatment. BNCT destroys cancer cells by fission products formed when thermal neutrons interact with nonradioactive ^{10}B isotopes delivered in cells with a neutronsensitizer. PDT kills cancer cells with reactive oxygen species (ROS) produced during activation of photosensitizer with light. A short free path of boron fission products (α particle, ^7Li nucleus) and ROS predefines selective damage of the cells, where neutron- or photo- sensitizer accumulates, whereas high intracellular concentration of the sensitizer assists in overcoming a defense action of antioxidative and reparative cellular systems. Efficiency of both BNCT and PDT depends critically on the properties of neutron- and photosensitizers, respectively, and new improved sensitizers are required.

Here we report on the development of a universal sensitizer being suitable for both PDT and BNCT: chlorin e_6 conjugated with boron nanoparticle by a flexible linker (Fig. 1). Chlorin e_6 is a recognized photosensitizer that is already used in PDT. The nanoparticle is an anionic cobalt-bis(dicarbollide) bearing 18 boron atoms. The linker consists of diaminohexane and diethylene glycol moieties. Different nanoconjugates have been synthesized and studied by us before an optimal structure of the sensitizer was found [1-5].

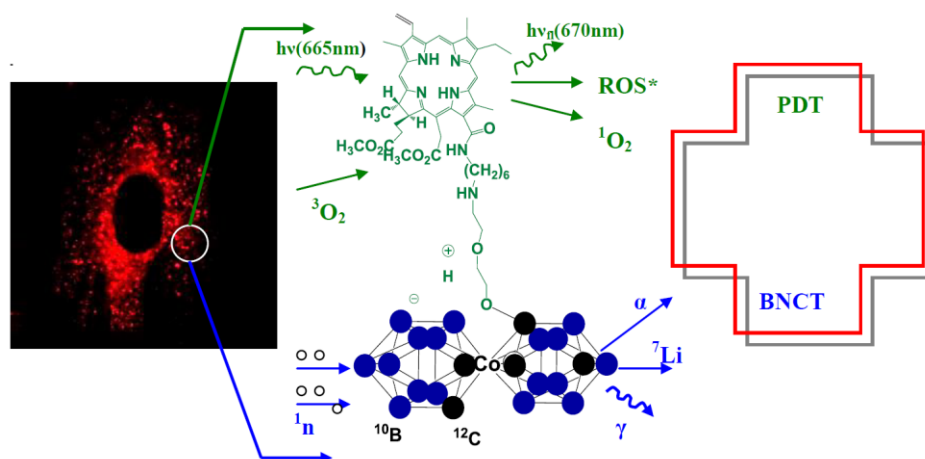


Fig. 1. Chlorin e_6 conjugated with cobalt-bis(dicarbollide) nanoparticle and its prospective applications

The developed conjugate has extremely high ability to accumulate in cytoplasm of different cancer cells including rat C6 glioma, human A549 adenocarcinoma, MCF7 breast adenocarcinoma and HeLa cervical carcinoma cells. Lysosomes are the sites of its enhanced intracellular accumulation. A

distribution ratio (the ratio of intracellular to extracellular concentration) of the conjugate depends on a cell type and varies from 50 to 80. The conjugate has reduced but still noticeable ability to accumulate in multidrug resistant MCF7R cells (distribution ratio of 9). It is characterized by fast cellular uptake and delayed efflux.

The conjugate is suitable for BNCT because it delivers $>10^9$ boron atoms per cell. According to a theoretical estimation *ca.* 10^9 boron atoms per cell is required for efficient BNCT. The conjugate is not toxic to cells without activating stimulus (neutrons, light). It possesses very high photoinduced cytotoxicity. Depending on the type of cancer cells photodynamic LD_{50} varies from 65 to 95 nm (780 nm for MCF7R cells). The conjugate fluoresces in a red region (670 nm) that is useful to monitor its accumulation and distribution *in vivo*. Properties of the conjugate warrant its preclinical evaluation as a multifunctional (theranostic) agent for BNCT, PDT and fluorescent tumor diagnosis.

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TESTING IMPACT OF NANOPARTICLES ON CELL PROLIFERATION: TRICKS AND TRAPS

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Abstract. Researchers are increasingly developing nanoparticles for biological applications. Before envisaging *in vivo* applications, it is of critical importance to test their effects on cell survival and proliferation. There exist numerous traps. Our presentation will summarize and give examples of traps to avoid and precautions to take, to efficiently measure the effect of nanoparticles on cells. Results show that air contamination by NPs, the effect of charges on intracellular localization of NPs, the effect of cell division and of trypsin digestion require specific precautions when testing the effects of NPs on cell survival and proliferation. Traps are not difficult to avoid but it is necessary to have them identified.

Although the potential of nanoscale objects in biology is tremendous, questions concerning the safety of nanomaterials and the risk/benefit ratio of their usage remain open. The first steps of NPs safety evaluation can be tested *in vitro* on cell proliferation and survival.

We chose silicon carbide based NPs (SiC NPs) according to the list of their advantages such as few nm size, bright fluorescence, easy solubilization in various polar liquids, longterm stability in solution, high cellular uptake, natural targeting of cell nucleus. There are spherical-like NPs with dimensions below 5 nm with the most probable size value being around 2.5 nm. Typical photoluminescence spectrum of these NPs dispersed in aqueous suspensions can be observed under ultraviolet excitation. SiC based NPs were found to be easily uptaken by living cells without any additional surface functionalization. Thus, they can be used as efficient fluorescent biomarkers.

Air contamination

When NPs have been initially dispersed in liquids, they may be easily transported into the surrounding air environment at relatively long distances due to the natural evaporation of the liquids at room temperature. Such an innate transfer of NPs from the originally colloidal suspension into an aerosol state may easily lead to a significant labeling of any biological system, although it is separated from the colloidal source of NPs, by a gas phase. These results should be taken into account in order to (a) avoid any contamination of control samples and (b) improve health protection of users. As a counterpart, NPs air transport may be used for soft cell labeling through the vapor phase.

Effect of cell division

The cellular uptake of NPs can be influenced by numerous factors, some of them are rather obvious, such as: NPs size, surface shape and composition of proteins on the surface of NPs, others are more difficult to extrapolate. Most of the study of NPs on cells are realized on proliferating cells. Indeed, when cells are isolated, they proliferate until they reach confluence, all the surface is occupied by cells, cells touch each other. At that stage, normal cells stop to divide while cancer cells continue to proliferate. When control cells are exposed to SiC based NPs, we observed a preferential localisation inside the nuclei. For a long time we have considered that result as normal, until we exposed control cells at the confluence to SiC based NPs. Under these conditions, SiC NPs do not enter inside nucleus and remain localized in the cytosol of cells. We performed complementary studies using cancer cells, these cells continue to proliferate even after having reached the confluence, that is a characteristic of cancer cells. Whether cancer cells are at the confluence or not, SiC based NPs are always localized inside the nuclei of these cells. The conclusion is that the intra nuclear localization of SiC NPs is dependent of the cell state. When cells are under conditions allowing cell proliferation, SiC NPs can reach the nucleus which is not the case when cells cannot divide. This suggests that NPs should be tested on both dividing and non-dividing cells.

Effect of peripheral charges

Peripheral charges of the NPs are known to influence their interactions with cells. SiC based NPs surface is naturally covered mainly by carboxylic acid groups. Covalent grafting of aminogroups

on the carboxylic acid functionalities, naturally covering the surface of fluorescent silicon carbide (SiC) quantum dots (QDs), allowed tuning of their surface charge from negative to highly positive. It has been possible to produce negative, neutral or positive SiC based NPs. According to their surface charges, the intracellular localization of SiC based NPs is different. Indeed, negatively charged SiC based NPs concentrate inside the cell nuclei, when close to neutrally charged SiC based NPs are present in both cytoplasm and nuclei while positively charged SiC based NPs are present only in the cytoplasm and are not able to move inside the nucleus. Results show that the electrostatic charges enable an efficient targeting in different intracellular compartments either the cytosol or the nucleus. These results open wide perspectives for discovering and understanding nucleus and cytosol transport mechanisms.

Effect of trypsin

In the vast majority of publication researchers show the effects of nanoparticle on few cells. This has two implications: first, it means that cells are proliferating (see the paragraph on cell proliferation) and secondly that cell have been exposed recently to trypsin. Trypsin is an enzyme often used to detach adherent-cell subculture from the substratum before reaching confluence. Cell suspensions are subsequently diluted and reseeded at lower concentration into fresh cultures. Trypsin digest adhesion proteins that keep the cells attached to the substrate. However, the proteolytic activity of trypsin may harm cells by cleaving the cell membrane proteins. When cells are exposed to SiC based NPs within 24 hours after trypsinization, we observed a maximal uptake of SiC based NPs and the absence of a dose-response effect. The uptake of SiC based NPs is markedly reduced when cells are kept 48 hours after trypsinization and a dose-response can be observed. These results suggest that trypsin digestion produces leaky plasma membrane which increases NPs uptake. A simple way to prevent that misleading result is to expose cells to NPs at least 48 hours after trypsinisation.

In conclusion, testing the effect of nanoparticles on cells is not so simple. Traps can be easily avoided, nevertheless it is important to identify them.

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LASER ABLATED SILICON NANOPARTICLES FOR BIOMEDICAL APPLICATIONS

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Abstract. We employed direct laser ablation in water and preparation of water suspensions from nanoparticles obtained in gas medium. These nanoparticles are ideal for biomedical applications due to the contamination free procedure. Nanoparticles obtained in gas (He) have high quantum yield of photoluminescence, so we demonstrated their bioimaging applications in living cells. Samples of another type were made in water by ablation of floating silicon microparticles instead of standard silicon wafer. This gives us a possibility to control final diameter (2-20 nm) of nanoparticles by changing initial concentration of microparticles.

Silicon nanoparticles (SiNPs) are very promising in biomedical applications due to their biocompatibility and biodegradability [1]. Possible applications are photosensitizers for photodynamic cancer therapy, bioimaging, drug-delivery containers and ultrasonic therapy. There are several methods of formation of SiNPs, e.g. electrochemical etching, chemical deposition, metal-assisted etching and laser ablation. The latter method has some advantages, i.e. its contamination free procedure, since it doesn't involve toxic chemicals or acids, and a unique possibility to obtain standalone nanoparticles despite highly agglomerated porous silicon nanoparticles. Dispersion and average diameter of SiNPs may be controlled by varying laser light power density, medium, etc. We report on formation of SiNPs ranging in size from 2 to 100 nm.

SiNPs is well-known to possess photoluminescence (PL) with quantum yield about 5% [2]. This is explained by the quantum confinement effect of charge carriers in small (less than 5 nm) nanocrystals. PL of SiNPs can be successfully used for bioimaging, because SiNPs emit light in the red and near infrared range of the spectrum [3]. Therefore the main purpose of this research is investigation of PL properties of SiNPs, and optimization of the formation procedure. We were mainly interested in the characterization of water suspensions, because it is the best form for application in biomedicine.

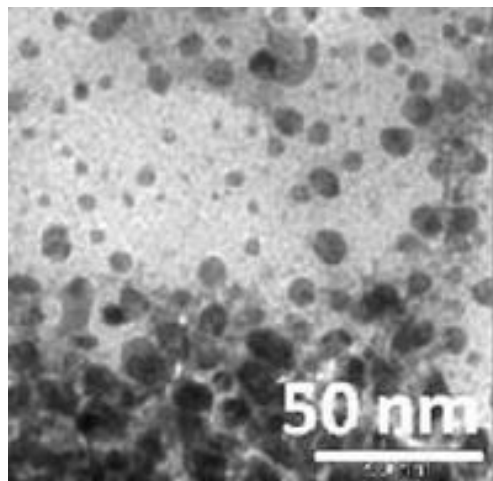


Fig. 1. TEM-image of silicon nanoparticles obtained by laser ablation in liquid

We employed both direct laser ablation in water and preparation of water suspensions from nanoparticles obtained via ablation in gas medium. Nd:YAG (wavelength = 355 nm, pulse duration ~ 40 ns, power density ~ 40 J/cm²) and Yb:KGW (1025 nm, 480 fs, 1 J/cm²) lasers were used for ablation. In some cases chemical etching was additionally employed for further reduction of SiNPs size [4, 5].

A typical transmission electron microscopic image is shown in fig. 1. Apparently, nanoparticles of this type can spread through organism via blood vessels and penetrate into living cells.

We compared two methods of SiNPs formation in liquids. The first method was laser ablation of silicon wafer (target) located in chloroform and the second one was laser fragmentation of silicon microparticles suspended in water, which were obtained by mechanical grinding of silicon wafers. Average size of microparticles was about 0.5 micrometer. In the first case we detected PL related to defects in SiO₂ due to its high emission energies (up to 3 eV). This was found for oxygen saturated suspensions. Ablation in oxygen free suspensions didn't give any significant PL.

Similar results were found for particles ablated in water. Spectra of untreated samples (suspension of microparticles – 1), samples obtained in oxygen saturated water (3) and oxygen depleted water (2) are shown in fig. 2. One can see a significant increase of PL intensity of SiNPs because of oxygen saturation. Note that SiNPs emit light in the red and near infrared range of the spectrum. PL of SiNPs may be explained in the framework of excitons confined in silicon nanocrystals.

The fragmentation technique has the following advantage: final average size of nanoparticles can be tuned by initial concentration of microparticles. Thus, the change in concentration from 0.1 g/L to 0.5 g/L gives an increase of the average diameter from 3 to 20 nm.

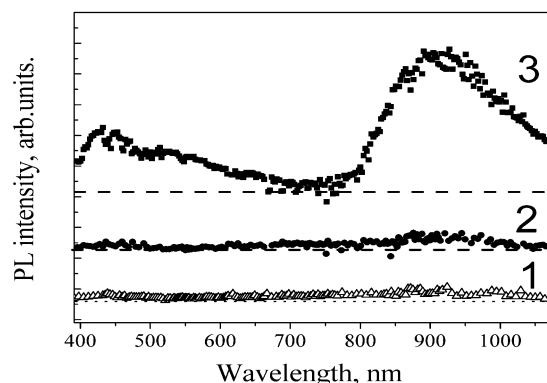


Fig. 2. Photoluminescent spectra of silicon microparticles (1), and silicon nanoparticles obtained by laser fragmentation in deoxygenated (2) and oxygen saturated water (3)

SiNPs created in liquid medium have a relatively low quantum yield of PL, possibly due to nonequilibrium cooling after ablation. Therefore we also tried to prepare suspensions from SiNPs created by laser ablation in gaseous ambient (He, 4 Torr). These suspensions had a relatively high quantum yield (about 1%), but an additional procedure was required for preparation of water suspensions from SiNPs layers deposited on a substrate.

Finally, we tested the obtained SiNPs for bioimaging application. Penetration through a plasma membrane was demonstrated. SiNPs provided high contrast in comparison with autofluorescence.

Thus, photoluminescent properties of silicon nanoparticles obtained via 3 different methods of laser ablation were investigated. It was found that the presence of oxygen in liquids provides necessary oxidation and leads to an increase in photoluminescent intensity. A new method of laser fragmentation gives new possibilities to control the size of silicon nanoparticles through changes of initial concentration of suspended microparticles. The obtained nanoparticles were successfully tested as luminescent labels in bioimaging application *in vitro*.

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LUMINESCENT NANORUBIES FOR BACKGROUND-FREE IMAGING IN CELLS

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Abstract. Molecular-specific luminescent probes can provide direct and minimally invasive approach for optical imaging in cells and living tissues. Single-molecule imaging is of special interest for investigating complex biological processes and molecular trafficking at the subcellular level. Here we report on new luminescent nanomaterial termed nanoruby (NR) which is a promising contrasting agent for optical bioimaging applications. Molecular probes based on NR particles can provide ultrasensitive high-contrast optical imaging in cells due to autofluorescence background suppression by employing the time-gating detection scheme.

Photophysical properties of NR

NR represents a Cr^{3+} -doped aluminum oxide crystalline $\alpha\text{-Al}_2\text{O}_3$ (corundum) nanoparticle [1]. It exhibits a very narrow emission spectral band (< 4 nm) centered at 694 nm falling within the so-called biological tissue transparency window, i.e. the spectral region of minimum absorption and scattering of biological tissue. As we have found, NR emission was characterized by the high quantum yield of $\sim 25 \pm 2\%$, and long lifetime emission of 3 ms, which is much greater compared to that of biological intrinsic fluorophores measured as several nanoseconds (termed autofluorescence). The luminescence was eminently photostable, blinking-free due to multiple Cr^{3+} centers contributing to the luminescence signal, and exhibited no signs of photobleaching during our observation period of 30 minutes. Also, a combination of physical and chemical properties were found advantageous for biolabelling applications.

In this paper, we report on NRs produced by femtosecond laser ablation of ruby crystal characterized by the $\text{Cr}^{3+}:\text{Al}^{3+}$ atomic ratio of 1.3% as measured by inductively coupled plasma mass spectroscopy. The mean diameter of as-produced NRs determined by means of dynamic light scattering and transmission electron microscopy was found to be 17 nm. The saturation laser intensity was of the order of 10^4 W/cm² at the excitation wavelength of 532 nm. The photophysical properties of the NRs also appeared to be unaffected by the cellular environment demonstrating the potential of NR as a bioprobe.

Colloidal properties

The zeta-potential of the as-synthesized aqueous colloid of NR, which provided a measure of the nanoparticle surface charge, was $+35 \pm 5$ mV explaining excellent colloidal stability due to the strong electrostatic repulsion forces. This charge resulted from the ion complexation reactions of amphoteric Al_2O_3 with water molecules depending on pH conditions. At neutral pH of ~ 7 , NR acquires a high-positive surface charge where positively charged moieties (AlOH_2^+) predominate over negatively charged groups (AlO^-). Decreasing pH causes lower net charge (Figure 1) [2].

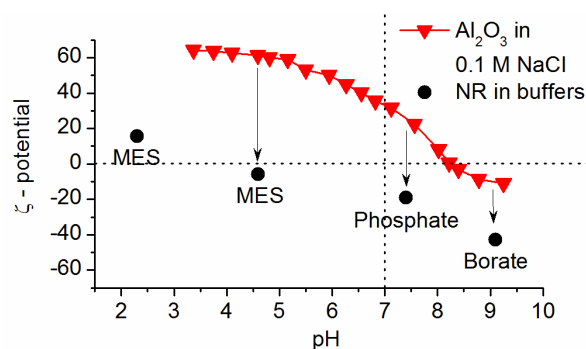


Fig. 1. Zeta-potential of nanoruby as a function of pH in various buffers, compared to that of Al_2O_3 in a non-buffered solution (0.1 M NaCl)

Besides, we measured NR zeta-potential in various anionic buffers, including 2-(N-morpholino) ethanesulfonic acid (MES), phosphate and borate showing negative shift of the zeta-potential (compared to that in water) due to the chemisorption of buffer anions on the positively charged AlOH_2^+ moieties, yielding excellent colloidal stability of the NRs in buffers. For example, dispersing NRs in phosphate buffer at pH 7.4 was accompanied by the following mono- and/or binuclear reactions of ligand exchange: $\text{AlOH}_2^+ + \text{H}_2\text{PO}_4^- \leftrightarrow \text{AlOPO}_3^{2-} + \text{H}_2\text{O} + 2\text{H}^+$ and $2\text{AlOH}_2^+ + \text{H}_2\text{PO}_4^- \leftrightarrow \text{Al}_2\text{O}_2\text{PO}_3^{2-} + 2\text{H}_2\text{O} + 2\text{H}^+$, respectively. The NRs demonstrated good biocompatibility with no significant effect on cells viability ($92 \pm 2\%$ versus $94 \pm 2\%$ in control sample).

Time-gated laser scanning confocal imaging

The extraordinary long millisecond-scale luminescence lifetime of NRs offers means to suppress the back-scattered excitation light and autofluorescence signals from the biological cells and tissue leading to greatly improved imaging contrast of nanoruby particles that can be used to tag biomolecules of interest. These means are realized by time-gated detection scheme, where a pulsed excitation is synchronized with the detection window, which was switched on 10 μs after the excitation pulse hit the biological specimen. As a result, the unwanted optical background was completely suppressed permitting detection of exclusively luminescent photons emitted by NR particles in the specimen. This approach was implemented in our home-built laser scanning confocal microscopy system, with the results shown in Fig. 2A. Chinese Hamster Ovary CHO-K1 cells with internalized NRs were non-specifically surface-labeled with quantum dots (QDs) and imaged in a conventional fluorescence mode, with broad detection spectral band (i), QD luminescence spectrally filtered (ii), time-gating imaging mode (iii). The time-gated imaging mode of NRs in as-grown cells resulted in complete (>20 dB) suppression of cell autofluorescence and hefty luminescence of exogenous QDs.

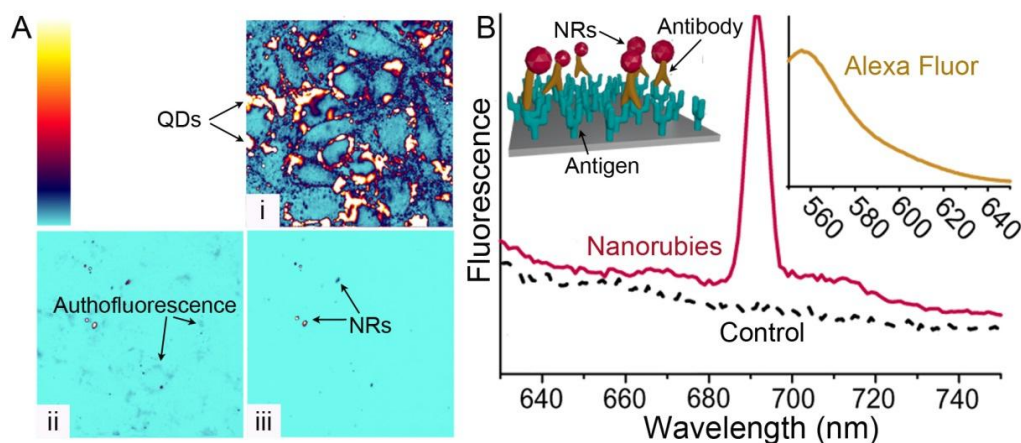


Fig. 2. (A) Time gated imaging of NRs in cells; (B) NR-assisted *in vitro* immunoassay

Immunoassay application

Immunoassay binding to target biomolecules was also performed (Fig. 2B). The high positive surface charge of the as-synthesized colloidal NR in water in the vicinity of neutral pH allows physisorption of negatively net-charged proteins such as IgG (pI 6.3–6.5). The Alexa Fluor 532-labeled goat-anti-rabbit IgG was attached to NR by simple mixing and specifically targeted a complementary antigen, rabbit IgG. The antibody-antigen binding was verified by measuring Alexa Fluor 532 and NR luminescence signals from the sample glass well, which showed no signal in negative control well without anti-rabbit IgG, demonstrating that our NRs are suitable for high-sensitivity facile bioassaying and lab-on-a-chip express biotests.

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OPTICAL PHOTON REASSIGNMENT MICROSCOPY

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Abstract. In the 1980s a concept was presented which acquires images of the pinhole plane of a confocal microscope at every position of the scan and the detected signal was computationally reassigned to a position half way between the detection and the nominal scan position. This concept of computational reassignment is now realized in an all-optical way. This has advantages in speed and noise efficiency. Theory as well as first experimental will be presented.

Introduction

The concept of a confocal microscope in which the pinhole is replaced with an array detector has been around for some time. In every position of the scanning laser beam, the image of the emitted fluorescence is detected. It has been shown that a preferable way of processing the so-obtained 4D data (scan position XY and detection position XY) is to assign the detected signal in the final image to a location placed half way between the nominal excitation and detection positions [1]. The reason is that this is the most probably position of the origin of the respective fluorescence. This results in an image, with enhanced resolution and detection efficiency, as compared to a standard confocal image. Recently this reassignment method has gained substantial attention [2], also in the framework of multi-spot excitation [3].

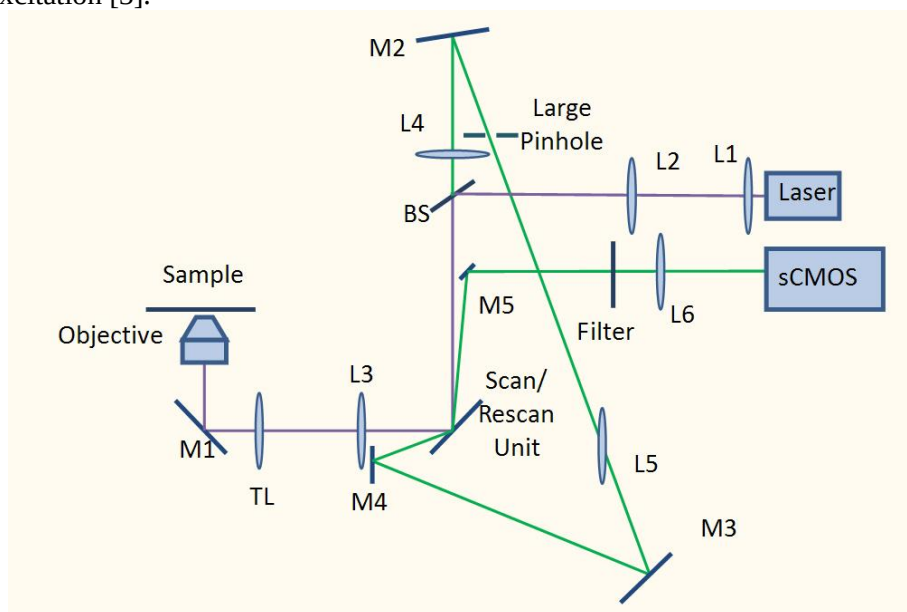


Fig. 1. Concept of optical reassignment: L4 and L5 have different focal length. All lenses are in 4f arrangement. (M: plane mirror, L: lense, BS: dichromatic beam splitter)

Method and Results

We here suggest ways which achieve a similar reassignment but in an all-optical way [4]. One possible realisation (Fig. 1) is to change the magnification between a de-scanning and a rescanning process. The beam enlargement in the parallel path leads to a reduced image of the pinhole plane on the CCD camera, whereas the re-scanning process and thus the nominal scan position on the CCD camera remains unaltered. In stark contrast to computational reassignment [1-3], it is now not necessary to acquire an image for every scan position. A single image yields the efficient super-resolved result.

Other ways to achieve a similar optical reassignment are to decouple the de-scan and re-scan process with the help of separate scan mirrors or to rescan twice. By tailoring the change in intermediate magnification, the system can be optimized for the influence of the Stokes-shift or even for realising a direct-view STED or RESOLFT microscope.

We discuss the theory of computational and optical reassignment and show experimental results. Concepts for realising optical reassignment for multi-beam scanners [5] are also discussed.

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BROADBAND SECOND HARMONIC GENERATION FROM GaAs NANOWIRES EXCITED BY HIGH POWER SUPERCONTINUUM FROM A PHOTONIC BANDGAP FIBER

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For years semiconductor nanowires (NWs) have been considered as potential building blocks for future nano-optical devices such as nano-lasers [1], frequency converters [2], and logic elements in nanoscale optoelectronic circuitry [3], due to their unique electrical and optical properties. The nonlinear optical properties, enhanced by the nano-level structure, are of significant importance for material research and optical applications. In this study, broadband supercontinuum (SC) pulses at 1000-1600 nm, covering the 1300-1500 nm region typically used in optical fiber communications, can also generate SHG signals with a bandwidth of 300 nm. Furthermore, to clarify the coherence of SHG signal, high polarization dependence on the femtosecond laser is detected.

Experiments and Discussion

GaAs NWs in experiments were grown by metal organic chemical vapor deposition on a GaAs (111) B substrate. The length of the NWs in growth direction was around 5 μm imaged by scanning electron microscopy (SEM) as shown in Fig. 1 (a). The distribution of their diverse diameters was centered at 160 nm as shown in Fig. 1 (b) ($n=170$). To excite the SHG signal, the supercontinuum laser was focused on NWs by an objective (40X, NA=0.65, Olympus) with a focus spot of 2 μm at room temperature with the beam approximately parallel to the NWs (Fig. 1 (c)).

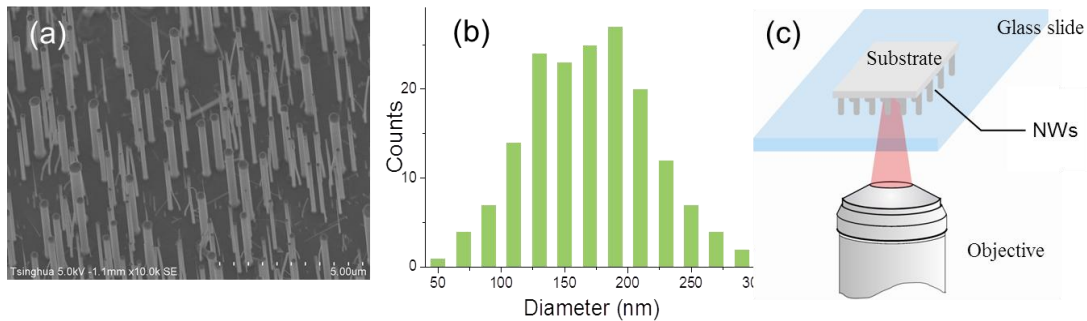


Fig. 1. (a) SEM images of GaAs NWs with a 20° view from surface normal direction view. (b) Distribution of diameters of NWs by random selection in the SEM images ($n=170$). (c) Optical scheme of SHG excitation by femtosecond lasers

By launching the femtosecond laser beam at 1040 nm into a 30-cm-long all-solid photonic bandgap fiber (PBGF), an output of SC laser ranging from 1000 nm to 1600 nm was acquired. Interestingly, after focusing this broadband beam onto the NWs, SHG signals from 500 nm to 800 nm were detected with a quadratic power dependence on the SC pulses as shown in Fig. 2 (a) and (b). Photons at different wavelength in the SC pulses excite SHG-corresponding signals simultaneously, indicating that the process is nonresonant (coherent).

To eliminate the possibility that the diverse diameters of NWs contribute to broadband SHG, NWs of uniform size were produced by Au aerosol seed particles as catalyst [4]. Those NWs sharing a 200-nm diameter were excited by the SC pulses as control. Again broadband SHG signals were detected as in Fig. 2(c). Hence the SHG signals were not a sum of resonant modes in NWs of different sizes, which again confirmed that the SHG was nonresonant.

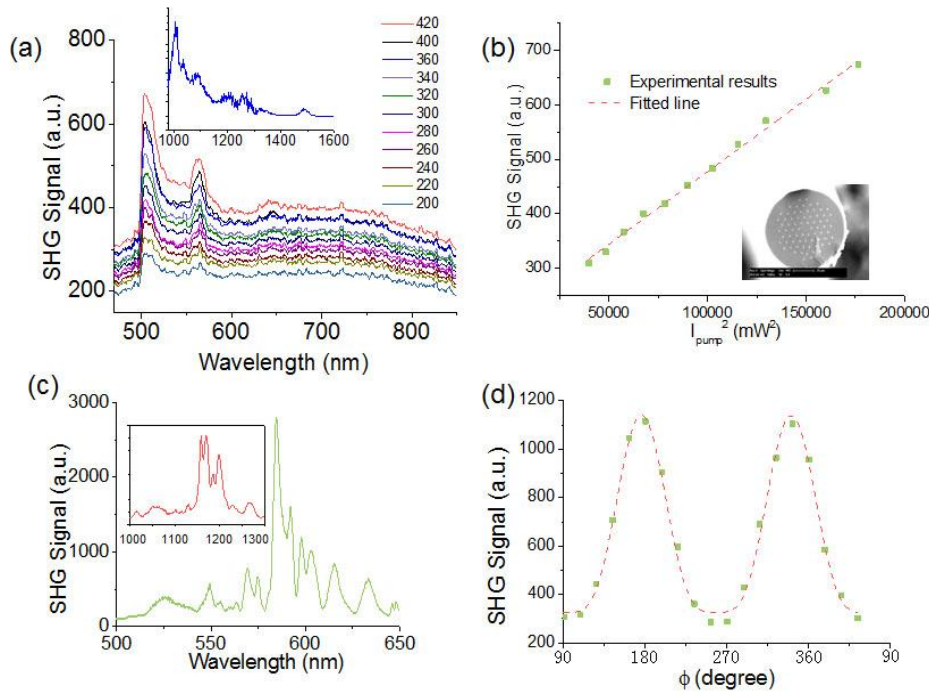


Fig. 2. SHG signals excited by SC pulses at 1000-1600 nm (a) at different powers (mW). The power shown in the figures was measured at the output of supercontinuum. Insert: spectrum of supercontinuum. (b) SHG signals had a quadratic power dependence on the pumping lasers. Insert: SEM image of the PBGF. (c) NWs with the same size could still generate broadband SHG signal excited by SC pulses. Insert: spectrum of SC pulses. (d) SHG signal had a polarization dependence on ϕ . Green dots: experimental results. Dashed line: $(\cos \phi)^4$ fitting

As coherent SHG depends explicitly on the crystal lattice structure of the NWs, high polarization dependence should be obtained [5]. To verify this point, the NWs were cut from the substrate and dispersed on a Si wafer which was less than one per $100 \mu\text{m}^2$. One single NW can be focused by a homemade femtosecond laser at 1040 nm, whose polarization was rotated by a half-wave plate. Setting the angle between the longitudinal axis of the NWs and the polarization as ϕ , the SHG signal was acquired as in Fig. 2 (d) showing the polarization dependence.

Conclusions

In summary, this study has shown the SHG excited from free-standing GaAs NWs by broadband SC laser pulses ranging from 1000 nm to 1600 nm. The SHG signal has a 300-nm bandwidth. The coherent SHG signal yields a high polarization dependence on a single NW. Our results suggest that GaAs NW is a potential broadband optical nonlinear converter and very promising for large bandwidth photonics and nanoscale optoelectronics applications.

Acknowledgment

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DISTRIBUTED SYNTHETIC GENE OSCILLATOR

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Abstract. Synthetic biology faces a substantial limitation in the complexity regulatory circuits that could be loaded in a cell. An additional complication is the reduced functionality and viability comparing to wild type strains. We investigate a promising way around these problems by distributing parts of a complex circuit among several cell populations and arranging interaction via quorum-sensing mechanism. We find that the mathematical model of coupled «activator» and «repressor» cell types demonstrates self-sustained oscillations in a wide range of relative cell concentrations.

Designing regulatory gene circuits that perform a target function is a central task of synthetic biology. One of the most challenging problems here is understanding and controlling complex dynamical regimes of such circuits. It is not only uncovering fundamental principles of gene regulation of natural networks that drives the research; much interest is spurred by the promises of biosensors and cellular machines for biofuels and organic waste recycling. While the recent years have seen an impressive success in building synthetic gene oscillators, toggle-switches, cellular communications and computing [1], one of the persistent limitations is the number of exogenous constructs one can make work in a cell. It has proved fairly difficult to realize more than minimal circuits, let alone integrating useful functionality on top of complex dynamics.

Recently, the potential of coupling cell populations with different circuitry to achieve more complex functionality has been noticed and implemented in sequential logic operations [2]. Here for the first time we propose a scheme for a distributed synthetic gene oscillator. The whole circuit is split into two cell populations, the «repressors» and «activators», communicating through the quorum-sensing mechanisms. Both types of cells are assumed to retain full viability; no apoptotic mechanism is involved, in contrast to the «predator-prey» model in [3]. Quorum-sensing employs chemical mediators (N-acylated homoserine lactones – AHLs), which exhibit selective sensitivity [2]. A specific AHL-family mediator is produced in a transmitter cell by a chemical reaction dependent upon a certain LuxI-family protein. Then AHL diffuses through the cell membranes and through the medium and is selectively bound by the specific corresponding LuxR-family protein on the receiver side. The LuxR-AHL complex acts then as a transcriptional regulator in the receiver cell.

The regulatory circuit is sketched in Fig. 1 and comprises cells types A (repressors) and B (activators). *luxI1* and *luxI2* stand for two different LuxI-family genes, AHL1 and AHL2 are the corresponding AHLs. The matching LuxR-family genes (not shown) are constitutively expressed. The intermediate *lacI* gene is introduced in cell type B to implement repression, because typically a LuxR-AHL complex acts as an activator. The gene expression in both types of cells can be monitored by reporter fluorescent proteins, for example, YFP and CFP.

We write down a simplified dynamical model in the dimensionless form:

$$\begin{aligned}
 \partial_t x &= \frac{\mu + r_\tau}{1 + r_\tau} - x & \partial_t y &= \frac{1}{1 + (l/L)^m} - y \\
 \partial_t l &= l_0 \frac{\mu + a_\tau}{1 + a_\tau} - l & \partial_t a &= bn_1 x - \gamma a + D\Delta a \\
 \partial_t r &= bn_2 y - \gamma r + D\Delta r
 \end{aligned} \tag{1}$$

The dynamical variables are the normalized concentrations: x and y – of the LuxI1 and LuxI2 proteins, l – of LacI in cells type B, a and r – of AHL1 and AHL2. The parameters are: $m=4$ is the LacI oligomer length, $\mu \approx 0.01$ is the leakage of promoters, γ is the decay rate of AHL (normalized to that of LuxI), D is the AHL diffusion constant, Δ is the Laplacian operator, index τ denotes the time delay induced by diffusion of AHL through cell membrane, in the intercellular space, aggregating also AHL and protein production and activation times. The value of l_0 is the saturated concentration of LacI with fully activated promoter, L is the sensitivity of a *luxI2* gene promoter to LacI. l_0 can be controlled, for example, by adjusting the copy number of gene *lacI*, and L – by concentration of IPTG.

The parameters b_1 and b_2 give the AHL production rates in a cell; n_1 and n_2 are the corresponding cell densities.

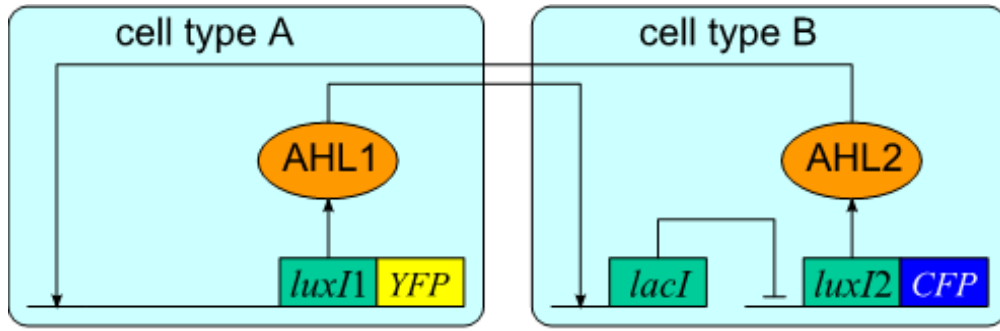


Fig. 1. Two populational distributed gene oscillator

Studying the spatially homogeneous dynamics of (1) we report the emergence of oscillations in a wide range of the model parameters. Interestingly, we find that they appear even under substantial imbalance between cell type A and B population densities (Fig. 2). Most striking, the density of activators could be only about 10% of the repressors to start oscillations. We propose that this effect can be used for sensitive early detection of a growing cell type in a colony of its abundant variant.

Spatially inhomogeneous dynamics is under current study.

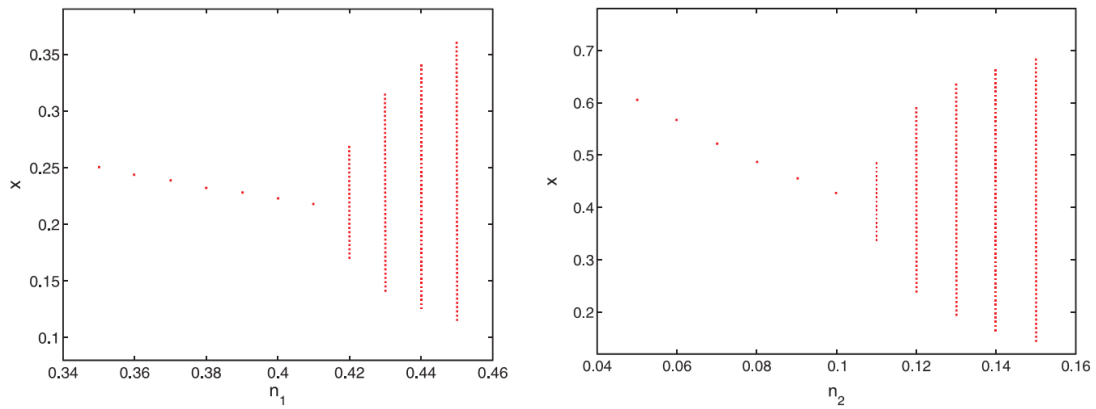


Fig. 2. Stroboscopic maps for LuxR1 concentrations recorded from numerical integration of (1) over $t=1000$ after some time left for transients to decay demonstrate emerging oscillations of gene expression. Left: increasing density of repressors, fixed density of activators $n_2=1$. Right: increasing density of activators, fixed density of repressors $n_1=1$. Parameters are $\tau=2$, $L=0.5$, $b=2$, $l_0=2$, $\gamma=1$

Acknowledgements

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THE USE OF SURFACE PLASMON RESONANCE BIOSENSORS IN BIOMEDICAL APPLICATIONS

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Abstract. Modern optical biosensors can monitor different intermolecular interactions in real time without any labels or associated processes. These devices have very high sensitivity, as their core technology is based on the effect of surface plasmon resonance (SPR). SPR provides kinetic, equilibrium and thermodynamic data in various biomedical applications: (1) analysis of any interactions between proteins, nucleic acids, carbohydrates, lipids and small organic molecules like drugs; (2) interactions of large objects (such as viruses, bacteria, liposomes, micelles and nanoparticles); (3) analysis of antibodies affinity; (4) affinity purification of target moleculless; (5) highly sensitive quantitative analysis of biomarkers.

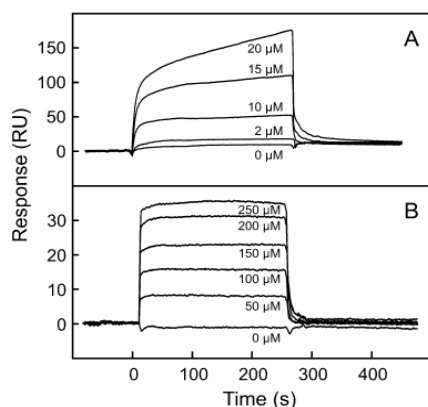
Introduction

The effect of surface plasmon resonance (SPR) related to the field of quantum nanooptics is utilized as a core technology in modern optical biosensors. These devices have very high sensitivity and can register almost all intermolecular interactions in real time without any labels or associated processes. SPR helps obtaining kinetic, equilibrium and thermodynamic data in various biomedical applications. Manufacturers of scientific equipment offer different SPR-biosensors with original design and functions. The best known models of biosensors are those of GE Healthcare (USA), Reichert Technologies (USA), SensiQ (USA), Bio-Rad (USA), Horiba (Japan) and others. However, the vast majority of biosensor research done in the world use the optical biosensor Biacore (GE Healthcare), which is due to two reasons: 1) Biacore was the first serial device equipped with a microfluidic flow system, and was widespread in various scientific and industrial fields; 2) Biacores have the best, extremely important parameters: sensitivity (about 10^{-11} M of analyte concentration); low noise (< 0.01 RU) and high stability (signal drift < 1 RU/h); no limitation on low molecular weight of analyte; low consumption of biomaterial (about 100 ng protein is sufficient); nano-flow cells (20 - 60 nL); valve switching topology of microfluidic flow system.

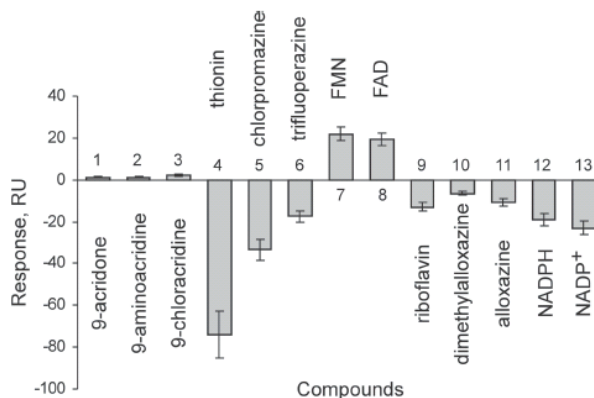
SPR biosensors in biomedical applications

There are four Biacore biosensors in the Institute of Biomedical Chemistry RAMS, which are used in biomedical research related to the analysis of various intermolecular interactions involving proteins, nucleic acids, lipids, and low molecular weight compounds. Some significant results were obtained in the following areas: discovery of protein dimerization inhibitors [1-8], protein-protein and protein-ligand interactions [9-11], proteomics and protein interactomics [12-16], protein-lipid interaction [17], DNA aptamers [18-19], amyloid- β peptide oligomerization [20-21], highly sensitive SPR analysis with signal amplification using gold nanoparticles [22-23].

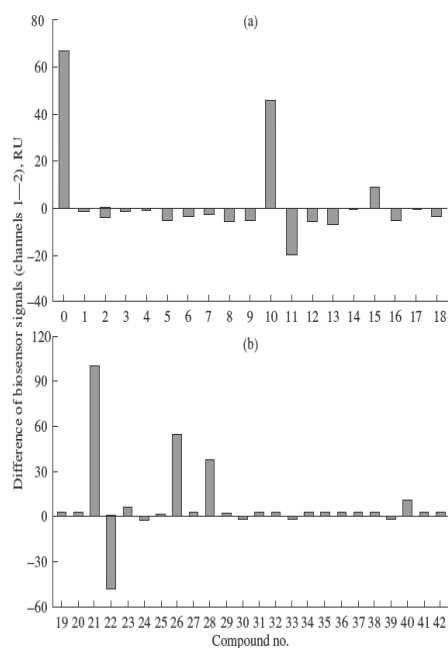
Some examples of SPR applications in our work are shown below.



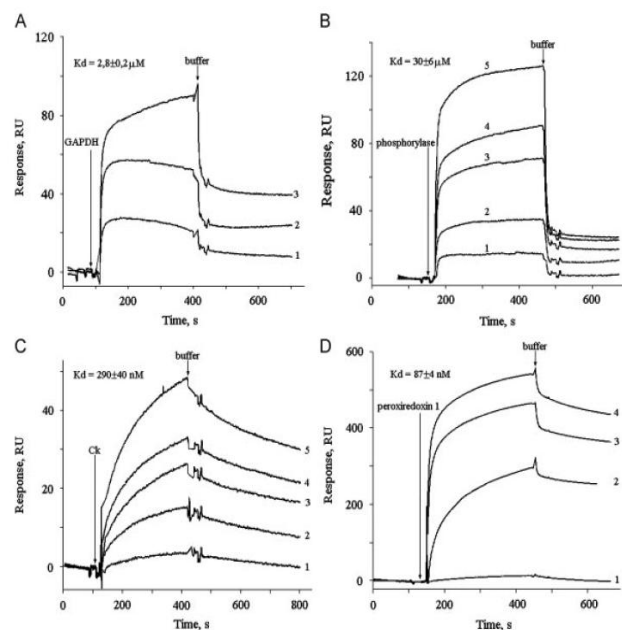
SPR sensograms of Aβ₁₋₁₆ (A) and Aβ₁₁₋₁₄ (B) interactions with immobilized Aβ₁₋₁₆ [20]



Small compounds binding to Cytochrome P450 Reductase [10]



SPR based screening of potential HIV protease dimerization inhibitors [6]



Isatin-binding proteins of rat and mouse brain: proteomic identification and optical biosensor validation [12]

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DISTRIBUTED SYNTHETIC GENE COMPETITION CIRCUIT

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Abstract. In spite of the impressive success of synthetic biology in recent years, the limitation of the synthetic network complexity to just a few genes per cell for technical reasons is still there. We propose an approach to get over this limitation by splitting the whole synthetic circuit into parts distributed among several cell populations. We implement this principle in the design of a synthetic gene model of competition. By means of analysis and simulations we find the emergence of local bi-stability, wave fronts and stationary structures in the model.

Synthetic biology is a rapidly developing field of research at the interface of biology and nonlinear physics. It is aimed at designing artificial genetic circuits with targeted dynamics and functionality. The growing interest in this topic is supported by anticipated applications, which include intelligent drug delivery, production of biofuels and biodegradation of organic wastes, as well as by the prospective fundamental challenge in understanding the principles of genetic regulation in natural genomes. The impressive achievements of synthetic biology currently include the implementation of various types of individual and collective dynamics (like switching, oscillation, synchronization, competition), logical operations, basic image processing tasks (see [1] for review). Still, the complexity of synthetic gene circuits is currently limited to just a few genes per cell due to technical constraints, like the issue of uncoupling with the cell's own gene circuitry. This also limits any payload gene functionality constructed on top of a synthetic regulatory circuit.

We propose a workaround to bypass this limitation by splitting the whole synthetic circuit into parts distributed among several cell populations. The overall complexity of the synthetic network can be increased this way, while the complexity of partial circuits in individual cells is kept achievable.

The intercellular communication can be organized by means of the quorum-sensing mechanism based upon the exchange between cells with dedicated chemical mediators (typically, N-acylated homoserine lactones – AHLs). In nature it is used by bacteria to adjust their behaviour in response to varying population density. A remarkable feature of this mechanism is selective sensitivity [2]. A specific AHL-family mediator is produced in a transmitter cell by a chemical reaction dependent upon a certain LuxI-family protein. Then AHL diffuses through the cell membranes and through the medium and is selectively bound by the specific corresponding LuxR-family protein on the receiver side. The LuxR-AHL complex acts then as a transcriptional regulator in the receiver cell. Due to the variety of the matched triplets of LuxI- and LuxR-family proteins and AHL-family mediators, the quorum-sensing mechanisms of different species in nature do not interfere. In synthetic biology this selective sensitivity can be used to establish multiple independent cell-to-cell signaling links.

The quorum-sensing mechanism is quite adopted by synthetic biology nowadays. However, it has been used mostly for communication within a single-genotype population, including such effects as synchronization of oscillations [3], spatial structure formation by an external AHL gradient [4], edges detection in images [5], and logical operations [6]. Applications to inter-population communication are still restricted to the specific task of population density control in ecosystem modeling [7]. We propose to use it for organizing intercellular connections in distributed genetic circuits.

We present a theoretical description of a genetic circuit design employing the distributed network principle. It is a synthetic gene model of competition between two species of cells. The species are of the same natural genotype, but are doped with different synthetic gene circuits. Each of the species produces and excretes its own type of AHL, which diffuses through the medium and inhibits the production of the opposite species' AHL. This leads to a competition between two genetic reactions in the corresponding cell species.

The genetic circuit design is depicted in Fig. 1, where *luxI1* and *luxI2* are two different LuxI-family genes, AHL1 and AHL2 are the corresponding AHLs. The matching LuxR-family genes are constitutively expressed, they are not shown in the diagram. The intermediate *lacI* gene is introduced to implement repression, because typically a LuxR-AHL complex acts as an activator. The gene expression in both types of cells can be monitored by reporter fluorescent proteins, for example, YFP and CFP.

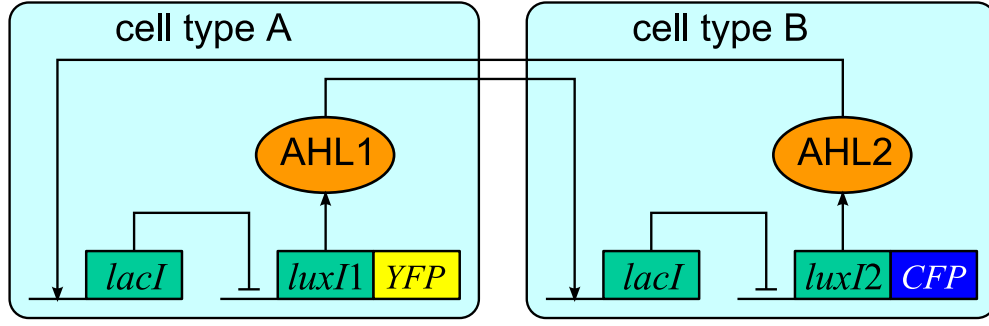


Fig. 1. Competition model design implementing the distributed network principle

We write down a simplified dynamical model of the network in the dimensionless form:

$$\begin{aligned}
 \partial_t x &= \frac{1}{1+l_1^m} - x & \partial_t y &= \frac{1}{1+l_2^m} - y \\
 \frac{1}{\gamma_2} \partial_t l_1 &= l_0 \frac{\mu+r}{1+r} - l_1 & \frac{1}{\gamma_2} \partial_t l_2 &= l_0 \frac{\mu+a}{1+a} - l_2 \\
 \partial_t a &= b_1 x - \gamma_3 a + D\Delta a & \partial_t r &= b_2 y - \gamma_3 r + D\Delta r
 \end{aligned} \tag{1}$$

The dynamical variables are the normalized concentrations: x and y – of the LuxI1 and LuxI2 proteins, l_1 and l_2 – of LacI in the two types of cells, a and r – of AHL1 and AHL2. The parameters are: $m=4$ is the LacI oligomer length, $\mu \approx 0.01$ is the suppression coefficient for inactivated promoter, $\gamma_2 \approx 1$ and γ_3 are the decay rates of LacI and AHL (normalized to that of LuxI), D is the AHL diffusion constant. The value of l_0 is the saturated concentration of LacI with fully activated promoter. This parameter can be controlled, for example, by adjusting the copy number of gene *lacI*. The parameters b_1 and b_2 control the AHL production rates and are proportional to the corresponding cell densities.

We start with analyzing the local dynamics of (1) at $D=0$ in the symmetric case ($b_1 = b_2 = b$). We derive, that under assumptions

$$\mu \ll \left(\frac{b}{\gamma_3} \frac{1}{l_0^m} \right)^{\frac{1}{m+1}}, \quad \frac{1}{l_0} \ll \frac{b}{\gamma_3} \ll l_0^m, \quad m > 1 \tag{2}$$

the underlying ODE system is in the bi-stable regime, which implies, that locally either of the competing genes can win, depending on the initial conditions. In the extended system, at $D \neq 0$, this local bi-stability ensures the existence of kink-type solutions, or fronts. We show, that in the symmetric case these fronts are immobile, while an asymmetry (for example, $b_1 \neq b_2$) leads to front mobility.

Numerical simulations confirm the mentioned analytic results, additionally showing, that coupling the gene dynamics (1) to populational dynamics can stabilize the fronts, leading to the formation of stationary spatial structures, if own mobility of cells is neglected.

The presented competition circuit design can be used for the experimental testing of the distributed gene network principle.

Acknowledgements

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MOLECULAR MODELING OF THE FÖRSTER RESONANCE ENERGY TRANSFER BETWEEN FLUORESCENT PROTEINS

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Abstract. Förster resonance energy transfer (FRET) is a powerful tool to investigate biochemical and biophysical processes *in vitro* and *in vivo*. It is applied to analyze changes in protein conformations, protein interactions and enzymatic activity. Fluorescent proteins used as FRET pairs are favorably employed as biosensors that can be targeted to organelles or tissues that permit monitoring not only of organelles and individual cells but also of entire organisms. We present the computational study of the FRET system – a fuse protein that is composed of two fluorescent proteins KFP and TagRFP joined with 23 aminoacid linker (TRK23). We analyze the origin of FRET properties of other known FRET pairs based on the fluorescent proteins and chromoproteins.

Introduction

Genetically encoded FRET (Förster Resonance Energy Transfer) biosensors that are based on the fluorescent proteins (FPs) and chromoproteins are becoming popular in cellular and molecular biology. Numerous processes in cells can be traced by using fluorescent proteins as *in vivo* markers for visual monitoring as FRET biosensors change their spectral properties, such as fluorescence lifetime or position of maximum of the emission band, while interacting with the target molecules. Moreover it is of great importance for the applications *in vivo* to design proteins with absorption and emission shifted to the red or near-infrared to reduce absorption, scattering, and autofluorescence at these wavelengths in tissues [2, 3].

Herein we present the computational study of the FRET system – a fuse protein that is composed of two fluorescent proteins KFP and TagRFP joined with 23 aminoacid linker (TRK23). We analyze all factors that influence FRET properties of TRK23. In addition, we compared other known FRET biosensors, such as FusionRed-Linker1-eqFP670, Caspr3(TarRFP and TagGFP fuse protein), mCherry-GFP and others to understand the origin of their properties and suggest the ways of their improvement.

Computational protocol

To analyze bound conformations of proteins we applied molecular docking procedure in ZDOCK program package starting with x-ray structures of monomers of TagRFP (PDB ID: 3M22) and tetramer of KFP (PDB ID: 1XQM) and chose top 500 structures. A combined quantum mechanics/molecular mechanics (QM/MM) approach was applied to find equilibrium geometry configurations on the ground state potential energy surface for KFP and TagRFP with the chromophores in anionic form (PBE0//cc-pvdz/AMBER). Equilibrium geometry configuration corresponding to the TagRFP emission was estimated in cluster model using multiconfigurational methods. Transition dipole moments for the absorption of KFP and emission of TagRFP were estimated at CIS//cc-pvdz (configurational interaction single) level of theory. Dynamics of unbound states was studied analyzing molecular dynamics (MD) trajectories of linker in water solution. 50 ns MD trajectory was simulated in NPT ensemble (T=300K p=1 atm) with 1 fs integration timestep. Calculations of MD trajectories were performed using the NAMD 2.6 software suite.

Results and discussion

TRK23 comprises tetramer of KFP as the core of the structure and four barrels of TagRFP at the periphery. Each monomer of TRK23 has a linker which is according to our calculations in the solvent-exposed conformation and has flexible structure.

We estimated possible bound structures applying molecular docking method and found all of them corresponding to the FRET efficiency values more than 60% that does not agree with the experimental observations. Thus, bound structures are not typical for this particular system. According to molecular dynamics simulation linker has a flexible structure and shows different conformations along MD

trajectory. FRET efficiency calculation of unbound structures gives the average result of approximately 50% that is in a good agreement with experiment.

Analysis of other FRET pairs shows similar results indicating that unbound structures are dominating for these fuse proteins.

Conclusions

Förster resonance energy transfer is a complicated process that depends on many different factors. Some of them depend on the nature of donor and acceptor species of the FRET pair, such as the overlap integral and the quantum yield of a donor. Other factors depend on the specific features of the 3-D structure of the proteins composing the FRET system: the distance between donor and acceptor pair and the orientation factor are the major components. Therefore, it is of great importance to carry out a comprehensive study of the system to understand which factors play the key role in the particular system.

We obtained complex modeling of the FRET properties of TRK23 and found them to be in a good agreement with the experimental observations. We suggest directions of improvement of FRET pair based on the comparative study of fuse protein.

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NOVEL FLUORESCENT PORPHYRAZINE MACROCYCLES AS FUNCTIONAL VISCOSITY PROBES IN LIVE CELLS

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Abstract. Here we report the results of the studies aimed at the development of functional materials for teranostics owing to the combination of their diagnostic and therapeutic capacities. Targeted materials are expected to be used as the fluorescent probes for local intracellular viscosity measurements as well as the efficient photodynamic therapy (PDT) agents.

Viscosity is one of the major parameters determining the diffusion rate of species in condensed media. It governs kinetics of bimolecular reactions and plays an important role in biological systems, as it influences on the rate of intracellular transport and signal transduction, depending on the hydrodynamic properties of the intracellular matrix, i.e. viscosity of individual domains within cells and the distribution of such domains. A common example is abnormalities in viscosity of cell membranes which were detected in patients with atherosclerosis, diabetes, Alzheimer's disease and even in cell malignancy. In spite of the intracellular microviscosity monitoring importance it continues to present significant challenges.

Series of novel fluorescent tetra(aryl)tetra(cyano)porphyrazine free bases and their metal complexes were prepared (Fig. 1).

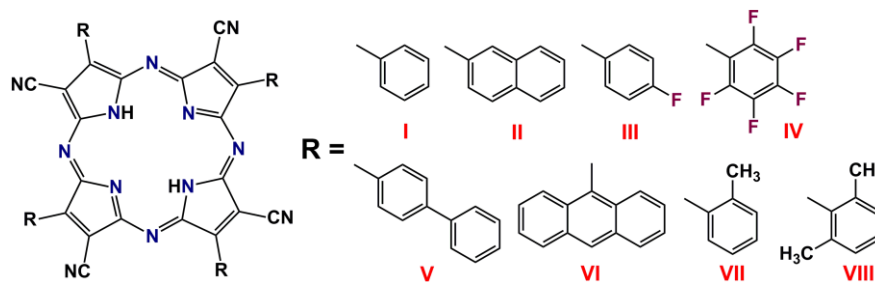


Fig. 1. Series of novel fluorescent tetra(aryl)tetra(cyano)porphyrazine free bases

The unique structural feature of these compounds is the alternation of peripheral strongly electron withdrawing and π -donor aromatic groups involved into a macrocyclic π -electron network (π -spacer). The synthetic fluorescent molecules consisting of 3 subunits (an electron acceptor unit, an electron donor unit, and a π -spacer) are known to demonstrate an the ability to undergo an intramolecular motion (rotation or twisting) in the fluorescent excited state [1]. Within this class of fluorophores termed fluorescent rotors intramolecular motion dominates the photophysical properties of the dye by changing the population balance between the radiative ("bright") and non-radiative ("dark") excited states. The intramolecular structural changes are typically viscosity dependent: in a non-viscous environment rotation is unobstructed and the population of the "dark" state is efficiently leading to low fluorescence. On the contrary, in a viscous environment the intramolecular rotation is slowed down and this leads to the increase in the population of the "bright" fluorescent excited state population. The benefits of this approach is gradual are that changing of the fluorescence parameters with viscosity are gradual and can be described by mathematical equations [2]. The relationship between dependence of fluorescence quantum yield (ϕ_f) and on viscosity (η) is described by the Förster-Hoffman equations [2]:

$$\phi_f = z \eta^\alpha \quad (1)$$

where z and α are constant. This equation can be readily modified for the fluorescence lifetime τ_f : [3]

$$\tau_f = z \eta^\alpha / k_r \quad (2)$$

where k_r is radiative decay rate constant.

So, the responses of the molecular rotors can be directly calibrated and viscosity can be directly measured by detecting the change in fluorescence parameters (quantum yield or life time) of molecular rotors. Thus, the Förster-Hoffman equation provides the framework for measuring viscosity in microscopic compartments of biological samples, using fluorescent molecular rotors. The cellular studies require the biocompatible rotor molecules to be available which are characterized by high uptake to cellular compartments of interest.

The compounds reported here are found to demonstrate a strong dependence of ϕ_f and τ_f on viscosity (Fig. 2). The plots of $\log \phi_f$ and $\log \tau_f$ as a function of $\log \eta$ for I-IV are yielded a straight lines with the slopes α changing in the range $1/3$ - $1/2$ that is very typical for fluorescent molecular rotors [3].

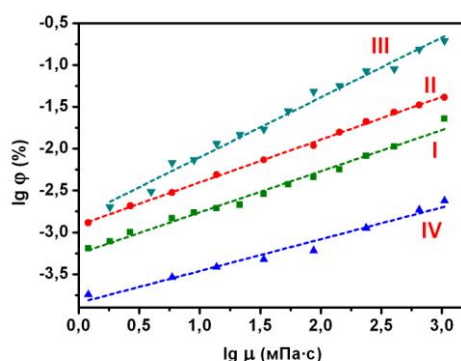


Fig. 2. Log fluorescence quantum yield of porphyrazines I-IV in ethanol/glycerol mixtures vs log viscosity in ethanol-glycerol mixtures

It was shown that that these novel dyes combine properties of the molecular rotors with high photodynamic activity (cell kill upon irradiation with visible light). In prospect that allows monitoring viscosity during real-time photodynamic therapy process to be investigated utilizing monitoring intracellular viscosity change. We confirmed that The spectral detection of singlet oxygen is indeed generated during the irradiation of the obtained free porphyrazine base with light at 660 nm was studied. Various polymeric nanoparticles incorporating novel chromophores added to the incubation medium containing human epidermal carcinoma A431 cells were investigated by scanning laser confocal microscopy method and lifetime images of porphyrazine base intracellular localization were had been obtained. It was established that the maximum income uptake of the photosensitizer into a cell was provided by the use of polyimide-*graft*-(polymethacrylic acid) regular polymer brushes as a solubilizer.. In this case the essential photosensitizer accumulation in the near-nuclear area washad been observed that is very beneficial for an efficient photodynamic therapy. The cell investigations confirmed a significant photodynamic activity of the synthesized compounds.

Acknowledgements

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3D-TUMOR SPHEROIDS AS A MODEL FOR TESTING PHOTODYNAMIC THERAPY WITH GENETICALLY ENCODED PHOTSENSITIZERS

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Abstract. Photodynamic therapy (PDT) is an effective method for cancer treatment. In preclinical studies of photosensitizers development of the technique that provides fast and inexpensive testing of the drug and treatment modes is crucial. Cell suspension and monolayer culture are the classical *in vitro* models, but they do not adequately reflect tumor properties. An alternative is 3D-culture, or spheroids. The purpose of our work was to create the tumor spheroids from the cells expressing genetically encoded photosensitizers KillerRed and miniSOG and assess the possibility of using such system for selection of the PDT mode before *in vivo* studies. In this paper we present the first investigation of the impact of laser radiation on growth and condition of the tumor spheroids and show that the 3D-culture is a promising tool for preclinical research of PDT with genetically encoded photosensitizers.

Photodynamic therapy (PDT) is an effective method for cancer treatment. A new prospective direction in PDT is genetically encoded photosensitizers. Recently, the ability of genetically encoded photosensitizer KillerRed to induce cancer cell death under light exposure has been shown *in vitro* and *in vivo* [1, 2].

In preclinical studies of photosensitizers development of the technique that provides fast and cost-effective testing of new drugs and treatment modes is crucial. Cell suspension and monolayer culture are the classical *in vitro* models, but they do not adequately reflect tumor properties, while *in vivo* animal tumor models are much more expensive, undergo strict ethical regulations, and require special professional skills.

An alternative is 3D-culture, or spheroids. Spheroids are three-dimensional multicellular structures measuring 50-1000 μm [3]. The structure of spheroids is heterogeneous. They have three main zones: necrotic core, quiescent viable cell zone and proliferating zone [4]. Use of spheroids for preclinical examination of chemically synthesized photosensitizers has been reported previously [5].

The purpose of this work was to create the tumor spheroids from the cells expressing genetically encoded photosensitizers KillerRed and miniSOG and assess the possibility of using such a system for selection of PDT mode.

The 3D-spheroids were generated from cell cultures of mouse colorectal cancer Colo26 with KillerRed and human cervical carcinoma HeLa Kyoto with miniSOG and non-expressing the proteins. The spheroids were cultivated in 96-well plate in Matrigel in conditions of CO₂-incubator, according to the protocol described in ref. [3]. The 3D-cultures were irradiated by means of the continuous laser ($\lambda=594\text{ nm}$ for KillerRed, $\lambda=473\text{ nm}$ for miniSOG, 150 mW/cm^2). Two treatment modes were tested: 1) irradiation on the 3rd and 5th day of the cultivation for 10 min, 2) irradiation on the 12th day for 20 min. The spheroids were examined on the inverted fluorescence microscope Leica DMIL from 3 to 27 days of cultivation. The fluorescence intensity and the size of the spheroids were measured.

We revealed that on the 3rd day of cultivation diameters of all spheroids were approximately identical (54 μm). In two days after laser irradiation (day 5 of the cultivation), the irradiated Colo26 spheroids with KillerRed were almost twice less in diameter (64 μm) than the irradiated spheroids without the protein (113 μm). Re-irradiation of the spheroids on the 5th day caused death of all cells with phototoxic proteins by day 7 of the cultivation. Irradiation of HeLa spheroids expressing miniSOG also led to their disintegration by the 7th day, indicating phototoxic reaction.

At the second treatment mode on day 12, the initial size of the Colo26 and HeLa spheroids was about 200 μm . As a result, after 20 minute irradiation the condition of the spheroids worsened by day 15. By the 18th day the KillerRed-expressing spheroids lost a clear boundary, became transparent at the periphery, while miniSOG expressing spheroids showed a tendency of disintegrating into cells. By day 21 all the spheroids were completely destroyed (Fig. 1). In the control 3D cultures without phototoxic proteins the dystrophic changes appeared on day 21, and their death came on day 27.

Photobleaching of KillerRed in Colo26 cells was observed in both modes. After a 20-minute laser irradiation its fluorescence did not recover.

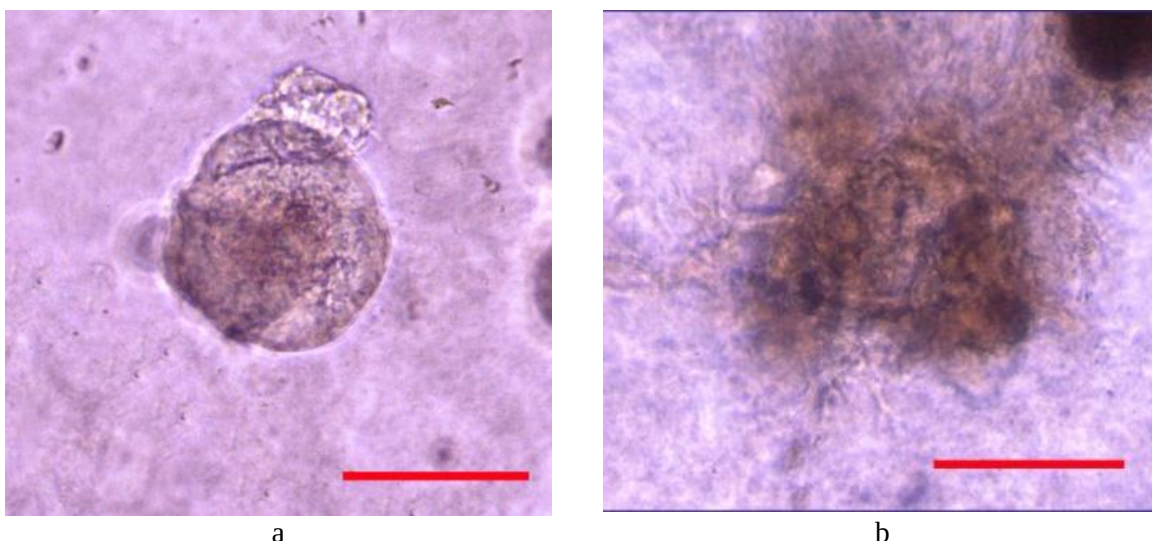


Fig. 1. Colo26 spheroids on day 21 of cultivation irradiated at 594 nm, 150 mW/cm²:
(a) without KillerRed, (b) KillerRed-expressing spheroid. Bar is 200 μm. Light microscopic images

Thus, we showed the phototoxic effect of fluorescent proteins KillerRed and miniSOG on tumoral spheroids. The results testify to the opportunity and prospects of the use of 3D-cultures as a model allowing to test treatment modes for PDT with genetically encoded photosensitizers.

Acknowledgements

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TOWARDS OPTICAL COHERENCE TOMOGRAPHY ENABLED FUNCTIONAL IMAGING

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Abstract. The object of biological imaging in three dimensions is to put function in its structural context and where possible to do this in a relevant living organism. This should be achieved with spatial and temporal resolution which are sufficient to observe the phenomenon of interest. We have developed several innovations in in vivo imaging in an effort to further this goal. Correlation mapping Optical Coherence Tomography (cmOCT) provides the functional information relating to the microcirculation, full-range OCT permits doubling the range while providing the highest sensitivity at the centre of the image and through the zero delay line. Finally, we have developed a scheme which permits miniaturizing OCT for deployment in primary care and other low cost applications.

Introduction

The most common functional information which can be observed in a living organism is its blood flow and oxygen status. This can be determined using Doppler methods, but this requires great phase stability and is difficult for angles between the laser beam and the flowing blood cells which are close to 90°. Alternative methods were developed based on speckle variance of the structural image intensity have been used in tumour microvascular imaging with high-frequency ultrasound [1] and more recently with OCT [2]. The inter-frame speckle variance (SV_{ijk}) images of structural image of the OCT intensity (I_{ijk}) may be calculated as follows [3]:

$$SV_{ijk} = \frac{1}{N} \sum_{i=1}^N (I_{ijk} - \bar{I})^2 \quad (1)$$

where N , the number of B-mode frames SV is calculated across; i , slice index; j and k , lateral and depth indices of the images; and $\bar{I}$, the average over the same set of pixels. Typically eight or more frames are required. This method reduces the sensitivity to the angle of red cell movement with respect to the laser beam. The calculated variance is in the range of $\pm\infty$ and is dependent on the chosen window size. There is therefore some difficulty with its interpretation and a dependence on *a priori* structural knowledge.

Optical micro-angiography (OMAG) is a recently developed technique reliant on endogenous contrast, which effectively separates the moving and static scattering elements of tissue to achieve 3D high-resolution blood flow images approaching that of conventional histology, allowing for in vivo perfusion assessment. In OMAG, spectral interferograms are modulated by a constant Doppler frequency (e.g. by a piezo-translation stage), thereby making separation of the moving and static scattering components within the sample feasible. The mathematical analysis involved essentially maps velocities moving into the tissue away from the surface into one image and velocities moving out of the tissue toward the surface into a second image by means of the Hilbert transform and subsequent fast Fourier transform [4].

Methods

To overcome these difficulties we developed correlation mapping OCT (cmOCT) which is an elegant solution requiring only to adjacent frames. The frames must be well within the lateral resolution of the system or better still they should be repeat B-scans in the same location before moving to take another pair of B-Scans. Areas which have a high correlation are therefore non-moving structures and the areas with low correlation are moving. Then the correlation between these two full-range OCT B-frames is determined by cross correlating a grid from frame A (average of frames 1&2) to the same grid position from frame B (average of frames 3&4) using the equation below:

$$cmOCT(x, y) = \sum_{p=0}^M \sum_{q=0}^N \frac{[I_A(x+p, y+q) - \bar{I}_A(x, y)][I_B(x+p, y+q) - \bar{I}_B(x, y)]}{\sqrt{[I_A(x+p, y+q) - \bar{I}_A(x, y)]^2 [I_B(x+p, y+q) - \bar{I}_B(x, y)]^2}} \quad (2)$$

Such simple analysis can give rise to exquisite images detailing all the smallest capillaries in the human body as per the examples in fig. 1.

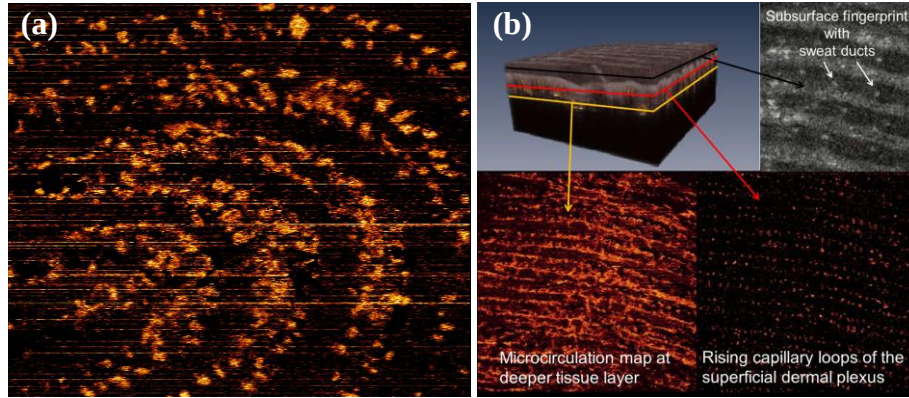


Fig. 1. a) Capillary loops following the pattern of the fingerprint and **b)** three levels of sub-surface fingerprint

A dual-beam OCT system [5] can be used to extract absolute velocity as shown in fig. 2.

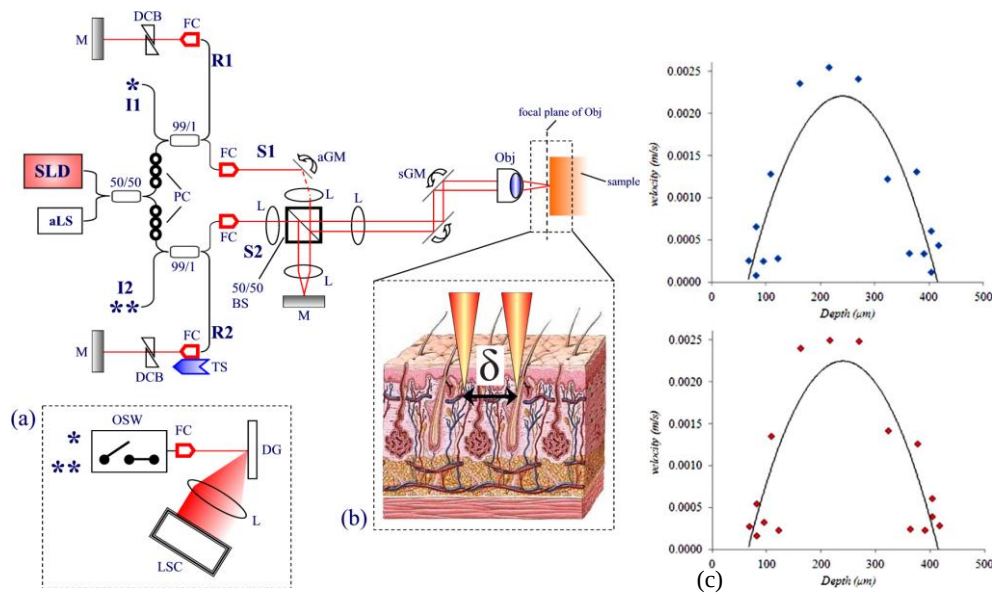


Fig. 2. Schematic diagram of the dual beam spectral domain OCT system setup. **a)** Depiction of the optical switch (OSW) mechanism used to alternately present each sample arm signal to the diffraction grating (DG) of a spectrograph and for detection by a line scan CCD camera (LSC). **b)** δ denotes the centroid separation of the sample beams, dictated by aGM movement. **c)** Depth-resolved flow profiles for (top) intensity and (bottom) phase based correlation, revealing laminar flow

Acknowledgements

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RAMAN SPECTROSCOPIC SIGNATURE OF LIFE CYCLE IN SINGLE UNICELLULAR ORGANISM (AMOEBA)

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Abstract. Raman spectroscopy was used to investigate the life cycle of Amoeba (*Acanthamoeba polyphaga*). Amoeba is a protist organism which plays an important role in the ecosystem and also can cause human and animals diseases. In the present work Raman spectra transformations corresponding to the Amoeba's various stages are analyzed. The spectra of a single Amoeba have been measured at different stages of the life cycle, including cyst formation (encystation) and excystation. The applications of Raman mapping technique for detection of the Amoeba in virulent state, and for development of rapid test of amebic infection are discussed.

Amoeba is protozoa microorganism dwelling in soil and water sources [1]. Several *Amoeba* species can cause diseases, including the ones leading to death of humans and animals, especially, *Naegleria fowleri*, *Acanthamoeba spp.* and *Balamuthia mandrillaris* [2]. Therefore, understanding of the *Amoeba* life cycle can be useful for amoebic infection prophylaxis, diagnosis and treatment.

Recently, laser technologies in bio-medical sciences are widely developed. The confocal Raman spectroscopic technique allows high sensitive imaging with high spatial and spectral resolution. Thus, it has become a useful tool for analysis of biological systems structure [3] and functions in biomedical researches [4]. Raman shifts can serve as a “fingerprint” for identification of the molecular composition and structure. The confocal Raman mapping can provide information about the distribution of chemical species in samples [5]. In this paper, we measure Raman spectra of the *Amoeba* at different stages of life cycle, analyse the shifting of spectra corresponding to the *Amoeba* transformations, and discuss the application of Raman spectroscopy and Raman mapping technique for diagnostics of *Amoeba* infections.

The free-living *Amoeba* representatives are the *Acanthamoeba*; we use *Acanthamoeba polyphaga* in our experiments. The *Amoeba* images during its life cycle were measured using confocal microscopy. The *Acanthamoeba polyphaga* has two main states in its life cycle: a trophozoite stage and a double-walled cyst stage. The *Amoeba* contains single vesicular nucleus and the nucleus has a densely staining nucleolus placed in the center. The size of *Acanthamoeba* in the trophozoite stage is 15-30 μm and the cysts size is 15 μm . When food becomes scarce or at environmental stresses, the *Amoeba* rounds up and encysts, these processes are called “encystation”. The *Amoeba* was grown in straw medium at room temperature; to simulate stress cold water with the temperature of 4°C was added to the culture dish and the *Amoeba* encystation process was observed. During this process the *Amoeba* loses its characteristic pseudopoidal movement and generates a protective wall. Once the appropriate environment was established, the cysts shed their coat and resumed trophic or vegetative life by the process called “excystation”. Straw medium was re-freshed to observe the cysts transformation to the mature cysts, and the trophozoite excystation from mature cysts was detected. Recent researches concerning the *Amoeba* life cycle are mostly focused on the encystation, and some characteristics for encystment process signaling pathways have been described [6-8]. However, the biochemical and molecular mechanisms involved in the transitions between cyst to trophic form and many pathways during this process are not fully studied.

In this work, we observe and analyze the *Amoeba* life cycles processes using Raman spectroscopy focusing at the stage of cysts transformation to mature cysts. At this stage, the *Amoeba* changes from a dormant state to an active metabolic state. The Raman spectra of the *Amoeba* in different states are shown in Fig. 1. The relative intensity of Amide I peak (near 1656 cm^{-1}) and signals from CH/CH₂ bonds in the 2900-3050 cm^{-1} range increased during *Amoeba* cysts transforming to mature cysts and the cysts activation. In the same time, the peaks at 1156 and 1519 cm^{-1} which can be attributed to carotenoid compounds decreased. We suppose that the observed changes are connected with structural transformations and chemical processes in membrane and transported processes in endosomal system in the *Amoeba* cysts.

The Raman mapping of *Amoeba* and its applications for the *Amoeba* state analysis as well as for the cell detection and infection diagnostics are discussed.

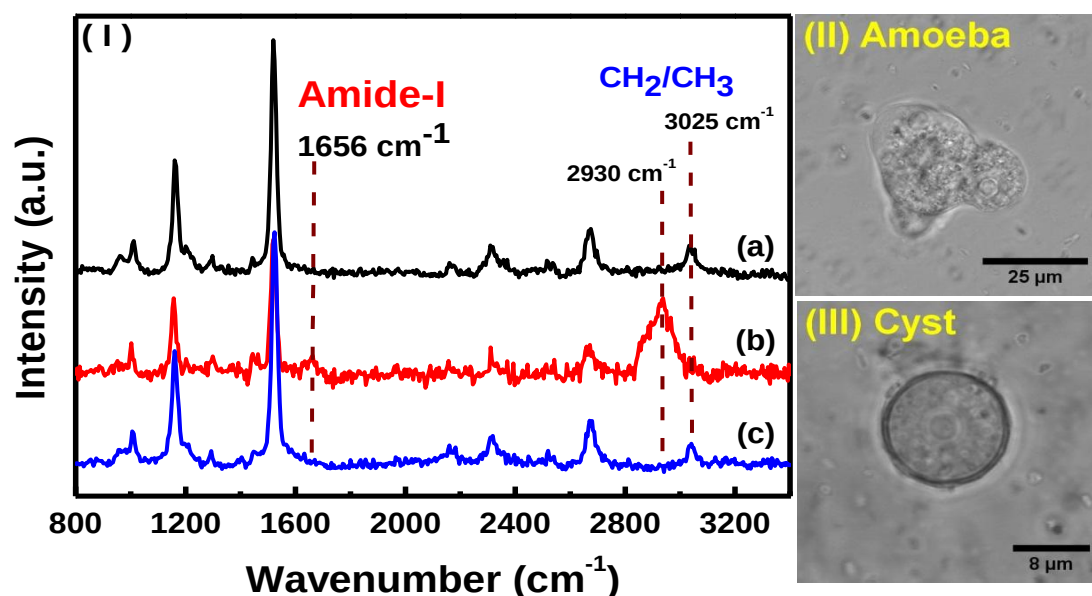


Fig. 1. Raman spectra of *Amoeba*, Mature cysts and Cysts: Raman spectra of (a) *Amoeba*, (b) mature cyst and (c) cyst, the Raman signal of *Amoeba* and cyst are, in the main, the same, Mature cyst has strong CH₂ signal (2930 cm⁻¹) and reveals the Amide-I (1656 cm⁻¹) Raman peak

Acknowledgements

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PHOTO-INDUCED CYTOTOXIC EFFECT OF 4D5scFv-miniSOG ON HER2/neu-OVEREXPRESSING CELLS

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Abstract. Here we report photocytotoxic anticancer effect of *de novo* designed genetically encoded immunophotosensitizer 4D5scFv-miniSOG. Recently a fluorescent flavoprotein miniSOG that efficiently generates singlet oxygen upon light illumination was described. We used this protein as a phototoxic module to design a targeted anticancer agent aimed to HER2/neu-overexpressing cells by mini-antibodies scFv. On SKBR-3 cell line we demonstrated that photokilling by 4D5scFv-miniSOG is highly specific and occurs only upon light irradiation. Moreover, our results state that co-treatment of these cells with 4D5scFv-miniSOG and mitotic inhibitor Taxol or non-toxic junction opener protein JO-1 produced a remarkable additive effect.

Introduction

The use of a fully genetically-encoded immunophotosensitizers (immunoPS) is emerging and very promising concept, which has been developing in our lab for a few years. We have recently designed a new protein 4D5scFv-miniSOG consisting of mini-antibody scFv specific to the HER2/neu receptor [1] and a new genetically-encoded protein miniSOG [2]. The resulting green fluorescent flavoprotein possesses the properties of both modules. It uses flavin mononucleotide (FMN) as a cofactor, upon blue-light illumination 4D5scFv-miniSOG generates singlet oxygen, excitation of the hybrid protein leads to green emission with two peaks at 500 and 526 nm. The K_D value of the 4D5scFv-miniSOG to HER2/neu receptor was determined to be 10 ± 2 nM.

The objective of the present study was to estimate 4D5scFv-miniSOG specific photo-induced cytotoxicity to adenocarcinoma breast cancer cells SKBR-3.

Results and discussion

In order to estimate the specific photo-induced cytotoxic effect of 4D5scFv-miniSOG on the viability of SK-BR-3 cells, a microculture tetrazolium test (MTT) was used. Decreasing concentrations of immunoPS were added to SK-BR-3 cells, and after 30 min incubation at 4°C and irradiation with bright white light (~ 1 W/cm²) for 10 min, the percentage of viable cells was determined compared to the untreated cells. Using regression analysis, IC_{50} was estimated as 160 nM.

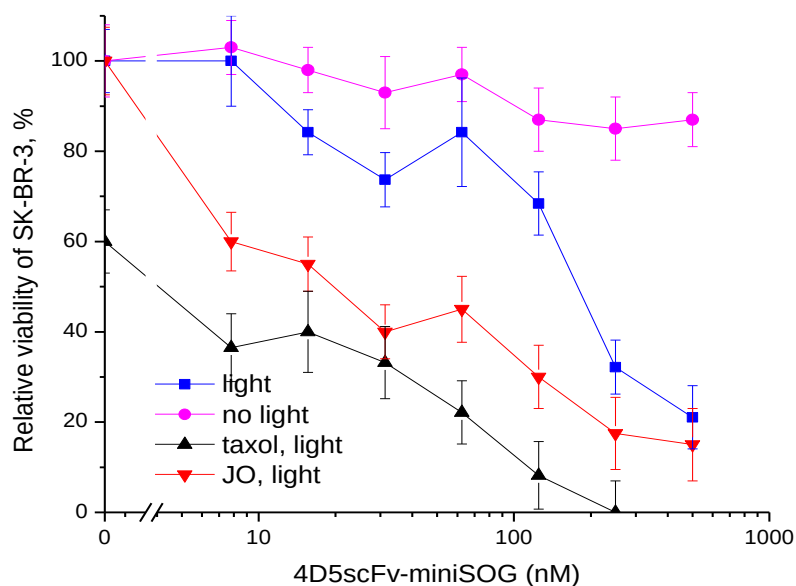


Fig. 1. Cytotoxic effect of 4D5scFv-miniSOG decreasing concentrations on the SK-BR-3 cell viability under different conditions: white light and dark, taxol or JO-1 co-treatment

We observed that the maximal cytotoxic effect (21% of viable cells) for SK-BR-3 cells was achieved at 500 nm of 4D5scFv-miniSOG (blue line).

ImmunoPS did not affect viability of the CHO cells in the dark or under white light even if treated with maximum concentration of immunoPS. Free 4D5scFv, free miniSOG and free FMN did not affect cell viability, indicating high specificity of the 4D5scFv-miniSOG phototoxic effect (data not shown).

We also investigated the cytotoxic action of 4D5scFv-miniSOG in combination with mitotic inhibitor Taxol (Paclitaxel) or junction opener protein JO-1. We showed that co-treatment of cells with immunoPS and Taxol leads to 100% SK-BR-3 cells death (black line), while complete cell killing was not achieved if 4D5scFv-miniSOG was applied alone (blue line). This strategy gives opportunity to use drugs in lower concentrations and decreases a possibility of side effects.

Another way for the reduction of immunoPS doses is to improve an access to its target receptor. It has been shown previously, that adenovirus junction opener protein JO-1 is capable of transient opening of intercellular junctions, leading to an increase of intratumoral penetration of the anti-Her2/neu monoclonal antibody trastuzumab [3]. We have found that JO-1 enhances immunoPS cytotoxicity, significantly improving the outcome of the breast cancer treatment with 4D5scFv-miniSOG (red line).

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RATIONAL SELECTION OF BACTERIAL PROTEINS FOR SPECIFIC BINDING OF FLUOROGENIC CHROMOPHORES

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Abstract. Fluorescent proteins and site-specific incorporation of fluorescent probes are state-of-the-art technologies for visualization of structures and processes in biological systems. One of the most promising new labeling methods is based on specific interaction of certain proteins and fluorogenic dyes due to expected high signal-to-noise ratio, photostability and other practical advantages. Here we report rational selection of such a pair from the library of synthetic GFP-like chromophores and short *E.coli* proteins with known structure.

Photostability of the probe is a major limiting factor for modern fluorescent labeling techniques. Higher photostability is expected in case of fluorogenic dye-protein pairs, due to continuous turnover of the dye. In comparison with conventional fluorophores, fluorogenic dyes are capable of increasing signal-to-noise ratio by reducing background fluorescence. Synthetic GFP-like chromophore and its derivatives are of particular interest as potential fluorogenic dyes. Fluorescence quantum yield of GFP chromophore approaches 1 within spatially restrictive environment of naturally-occurring fluorescent proteins. In water solutions synthetic GFP chromophore is characterized by about zero fluorescence.

We have synthesized a library of chromophores which are analogous to red chromophore of GFP-like protein Kaede with different substituents in position, corresponding to the first amino acid in chromophore-forming triade. In comparison with GFP-like chromophores, these chromophores possess substantial red-shift. Recent studies indicate the significance of chromophore's conformational lock for its fluorescence. To determine proteins with cavities capable of adapting Kaede-like chromophores in a tight manner and restricting its conformational freedom, we applied a custom docking algorithm for all available structures from *E. coli*.

Several most promising proteins were selected, cloned, expressed and purified. We observed selective reversible binding of chromophores with some proteins in vitro. As expected, in comparison with free chromophore, fluorescence intensity of chromophore-protein complex increased by more than an order of magnitude. These results demonstrate general applicability of computer-aided methods in design of fluorogenic labeling systems.

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IMAGING PIP₃ AND H₂O₂ WITH ONE GENETICALLY ENCODED FLUORESCENT SENSOR

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Abstract. We developed a genetically encoded fluorescent sensor (termed PIP-SHOW) for parallel measurements of phosphatidylinositol 3-kinase activity and hydrogen peroxide (H₂O₂) levels using two different types of readouts. Upon elevation of local phosphatidylinositol 3, 4, 5-trisphosphate (PIP₃) concentration, the sensor translocates from the cytosol to the plasma membrane, while a ratiometric excitation change rapidly and simultaneously reports changes in the concentration of H₂O₂. The dynamics of PIP₃ and H₂O₂ generation was monitored in platelet-derived growth factor-stimulated fibroblasts and in T-lymphocytes after formation of an immunological synapse. We suggest that PIP-SHOW can serve as a prototype for many fluorescent sensors with combined readouts.

Intracellular signaling events can be efficiently visualized using fluorescent proteins (FPs) based biosensors. Combinations of various fluorophores with distinct excitation and emission spectra in a single cell enable monitoring of multiple cellular processes simultaneously, usually termed multiparameter imaging [1]. Strategies allowing multiplication of imaged parameters in a single cell are highly demanded.

Phosphorylated forms of phosphoinositide lipids (PIPs) transduce signals via recruiting the PIP-binding domains that vary in their selectivity toward the number and position of phosphates of the inositol ring, allowing a fairly precise specificity of downstream signaling activation [2]. One important lipid messenger is phosphatidylinositol 3, 4, 5-trisphosphate (PIP₃). Phosphatidylinositol 3-kinase (PI3K) phosphorylates PI(4,5)P₂ to PIP₃, while the lipid phosphatase and tensin homolog (PTEN) reverses the phosphorylation [3]. FPs fused with PIP_n-sensitive protein domains allow monitoring of PIP_n formation by translocation of the fluorescently labeled domain from the cytosol to the plasma membrane (PM) [2].

The other recently recognized signaling system utilizes hydrogen peroxide, H₂O₂, as a second messenger molecule [4]. H₂O₂ selectively and reversibly oxidizes a small population of cysteines that tend to be deprotonated at physiological pH values [5]. H₂O₂ production by NADPH oxidase (NOX)/dual oxidase (DUOX) enzymes and generation of PIP₃ by receptor tyrosine kinase activation can be highly cooperative: Nox subunits p47 and p40 are recruited to the membranes by their PX domains that recognize products of PI3K activity and activate the Nox enzymatic activity [6]. H₂O₂ subsequently produced generates positive feedback loop oxidizing the active site thiolate of PTEN phosphatase increasing lifetime of PI3K products [7, 8].

Considering the interdependence of H₂O₂ and PI3K signaling, the simultaneous and spatially resolved measurement of both is desired in many cases. For this purpose, we have generated a sensor that reported changes in both PIP₃ and H₂O₂ concentration utilizing two different types of readout. Fusing fluorescent sensor for H₂O₂ detection (HyPer) [9] with a PIP₃-sensitive PH domain should allow visualization of both PIP₃ generation (by translocation of the probe from the cytoplasm to the PM) and of H₂O₂ generation (by monitoring the excitation ratio 500/420nm of HyPer). To generate a dual, PI3K and H₂O₂, sensor, we fused HyPer with a mutated PH domain (E41K) of Bruton's tyrosine kinase (BTK). The resulting reporter was named PIP-SHOW (PIP₃ and -SH Oxidation Watching).

In order to characterize the probe we expressed PIP-SHOW in NIH-3T3 fibroblasts and stimulated the cells with platelet-derived growth factor (PDGF). Addition of PDGF to NIH-3T3 cells caused both translocation of the probe to the PM and generation of H₂O₂. Notably, some amount of the PIP-SHOW always remains in the cytoplasm allowing comparison of the F500/F420 ratio between the PM and the cytoplasm. Addition of wortmannin, a specific PI3K inhibitor, to the pre-stimulated cells caused translocation of the indicator from the PM to the cytoplasm and inhibited H₂O₂ production. Nox inhibitor diphenyliodonium (DPI) led to a rapid drop in PIP-SHOW ratio reflecting decrease in H₂O₂ production.

Next, we utilized PIP-SHOW to study lipid and redox signaling events in the first phase of CD4⁺ human T helper (T_H) cell activation. Transient expression of PIP-SHOW in human T_H cells led to its

localization within the cytoplasm as well as within the nucleus. Stimulation of T_H cells expressing PIP-SHOW with anti-CD3/CD28-coated beads led to establishment of stable contacts, immunological synapses (IS), between T_H cells and the beads. This was followed by a massive and immediate redistribution of PIP-SHOW to the IS indicative of locally elevated PIP₃ levels. The probe remained in the contact region for the duration of the experiment (up to 1 h). Furthermore, IS formation also led to a rapid elevation of H₂O₂. Interestingly, in most of the cells H₂O₂ production was highest in the region adjacent to the central synapse, implicating a ring-like localization of NOX/DUOX enzymes around the central IS. DPI quickly attenuated H₂O₂ production, suggesting that PM-localized NOX/DUOX is the source of H₂O₂. Inhibition of PI3K by wortmannin, however, led to a rapid decrease in both PI3K activity and H₂O₂ production.

In summary, we validated PIP-SHOW using two cellular systems in which both PI3K and NOX/DUOX reactive oxygen species were already shown to be important determinants of several signaling pathways. We demonstrated that the novel dual-parameter sensor PIP-SHOW is well suited for simultaneous monitoring of PIP₃ and H₂O₂ levels and can thereby serve as a prototype for indicators with combined readouts. Our results provide relevant information about the effectiveness of the sensor but also new information about the cooperative dynamics of the two signaling systems.

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PRACTICAL ASPECTS OF STOCHASTIC OPTICAL RECONSTRUCTION MICROSCOPY (STORM) REALISATION

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Abstract. The present paper considers a subdiffraction localization fluorescence microscopy setup. This method allows one to obtain far-field images of biological structures, marked with fluorescent dyes with resolution up to twentieth part of emission wavelength. A method of compensation object's drift during data acquisition without need of additional hardware complications is discussed in details.

Fluorescence microscopy is a powerful tool for investigation of biological structures. Within the framework of this approach an object of interest should be marked with fluorescent dyes. The dyes are irradiated through an optical system of the microscope and emit fluorescence radiation with Stokes shift. This shift allows separating fluorescence from scattered excitation illumination and detecting it on the photodetector array. As every far-field imaging method, this method has a resolution limited by a diffraction barrier. This barrier follows from the wave nature of light. However, special behavior of fluorescent agents under illumination allows one to overcome this limit [1-4]. Among these methods we consider Stochastic Optical Reconstruction Microscopy. This method utilizes the property of some fluorescent dyes to be randomly switched on under certain illumination and environmental conditions [1]. The image of every fluorophore will be also limited by diffraction, but also spatially separated from another. This allows one to localize every fluorophore and build an image with resolution limited by localization accuracy. For the dyes emitting a large number of photons (e.g. Alexa 647), every fluorophore can be localized with an accuracy ten times higher than the diffraction limit.

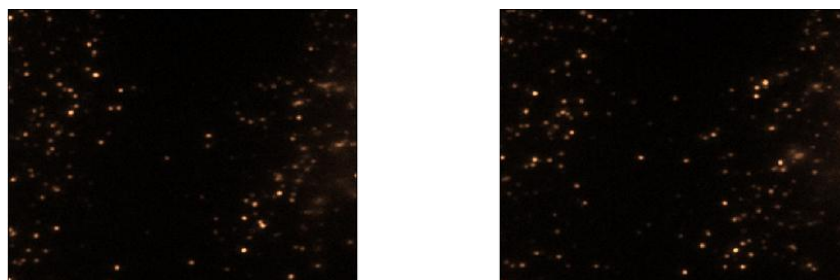


Fig. 1. Two sparse subsets of fluorophores detected in two different frames of EM CCD camera

This method, however, has some drawbacks. First of all, it is long time needed for data collection. One frame with sparse events consists of about 300 fluorophores, while for accurate image reconstruction 10^6 fluorophores should be localized. Considering EM CCD camera frame rate we can estimate image acquisition time to be several tens of minutes. With resolution at the level of tens of nanometers it gives us that the method is very sensitive to objects drift. The most common way to track the drift is to introduce several reference markers into the object. However, this makes sample preparation even more complicated. Note also, that according to the fact that every frame consists of different subsets of fluorophores, drift cannot be estimated with the use of cross-correlation. We introduce a method of obtaining and compensating the sample drift during STORM data acquisition which does not involve any additional hardware complications.

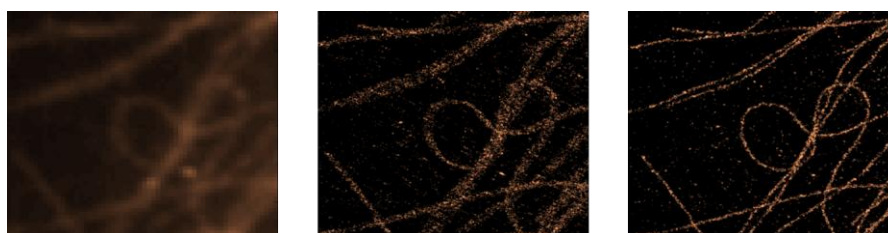


Fig. 2. From left to right. Conventional fluorescence image of biological structure, image, obtained with STORM technique before drift correction, image, obtained with STORM technique after drift correction

All experiments were made with STORM setup built in our lab with the use of Nikon Ti-E inverted microscope, Hamamatsu Image-EM EM CCD camera, laser illumination system developed in our lab as well as data processing software.

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SILICON NANOWIRES AND NANOLOGS FOR BIOPHOTONIC APPLICATIONS

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Silicon (Si) has a dominant role in electronics industry, but in fact it also can play important roles in biomedicine. For example, the biocompatibility and biodegradability of Si nanocrystals (nc-Si) and porous nanoparticles were demonstrated [1-3]. In particular, biodegradable nanoparticles of luminescent porous Si were proposed for the diagnostics *in vivo* [1]. Also *in vivo* experiments showed the biocompatibility of thermally oxidized porous Si films with the eye tissues, that can be used to improve existing therapies in patients with dysfunction of the corneal epithelial cells and ocular surface diseases [2]. Note, the total content of Si in the body of a healthy 70 kg adult is ~1 g, making Si the most important trace mineral in human body [3].

Silicon nanoforms as porous silicon (PSi) are promising for biomedical applications because of low toxicity combined with unique optical properties [4], and we propose that silicon nanowires (SiNWs) could have the same features. PSi and SiNWs can exhibit efficient room temperature photoluminescence (PL) in visible and near-infrared spectral range. The PL is explained by the presence of small silicon nanocrystallites (< 10 nm) in which excitons may appear due to quantum confinement effects [5, 6].

In our work luminescent SiNWs were obtained with Metal-Assisted Chemical Etching (MACE) of c-Si wafers (100) with specific resistance of 1 mΩ*cm (SiNWs) in 5M HF:30% H₂O₂ solution taken in 10:1 proportion, respectively. Etching continued for 30 minutes. The surface of Si plates was covered by Ag nanoparticles before etching. After the etching process Ag particles were removed by immersion into 65% HNO₃ solution for 15 minutes. Aqueous suspensions on silicon nanologs (SiNLs) were made by ultrasound milling (ultrasound bath) of SiNWs for 15 minutes in water. Structural properties of the obtained samples were investigated using the transmission electron microscope (TEM) of LEO912 AB OMEGA and the scanning electron microscope (SEM) of Lira Tescan. PL spectra were obtained under excitation of Ar-laser (448 nm). The cells containing SiNLs were studied using the confocal microscope Leica TCS SP5.

According to SEM and TEM data, the obtained SiNWs had an arranged structure of parallel wires with porous structure, which indicates the preferential orientation along the [100] crystallographic direction (Fig. 1a, b). Diameters of SiNWs vary from 20 to 200 nm.

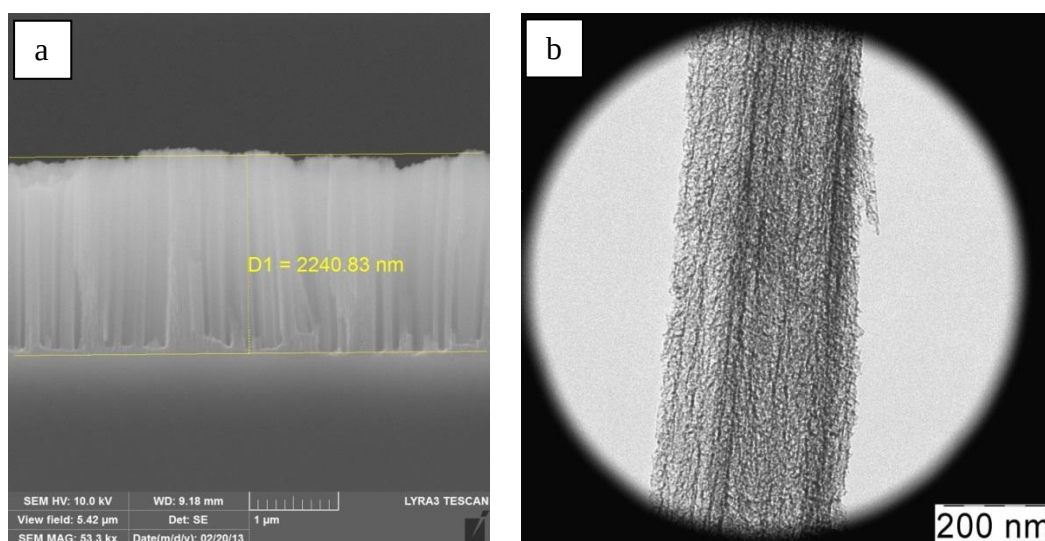


Fig. 1. SEM (a) and TEM (b) image of SiNWs

Typical PL spectra of SiNWs and SiNLs are shown in Fig. 2 a,b. The PL spectra of prepared samples had an appearance of a broad band in the visible range, with the maximum in the photon energy near 1.66 eV. PL of the samples may be explained as radiative recombination of excitons confined in small Si nanoparticles. Such Si nanoparticles may be in porous structure of SiNWs and SiNLs, and an average size of nanoparticles is $\sim 3\pm 0.3$ nm [3, 4].

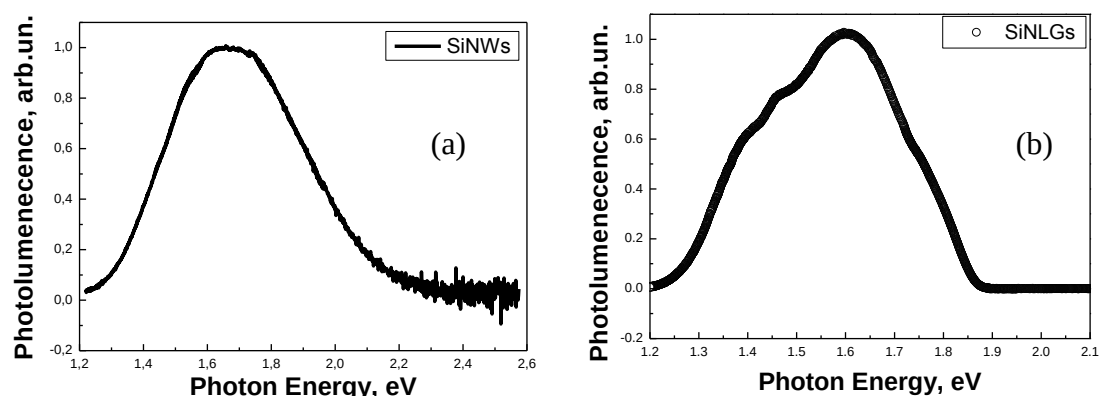


Fig. 2. PL spectrum of SiNWs (a) and SiNLGs (b)

In-vitro experiments revealed 50% cytotoxic concentration of SiNWs of about 0.4 mg/ml.

Figure 3 shows the luminescent photographs of living CF2Th cells with introduced SiNLs. Cell's cytoplasm (stained in green), cell's nucleus (stained in blue) and SiNLs (stained in red) shown are close to each other. It is evident that the majority of nanowires has penetrated into the cells due to the mechanism of endocytosis, and located in its cytoplasm and nucleus. The obtained results demonstrate that SiNLs can be used as luminescent labels in biomedical applications.

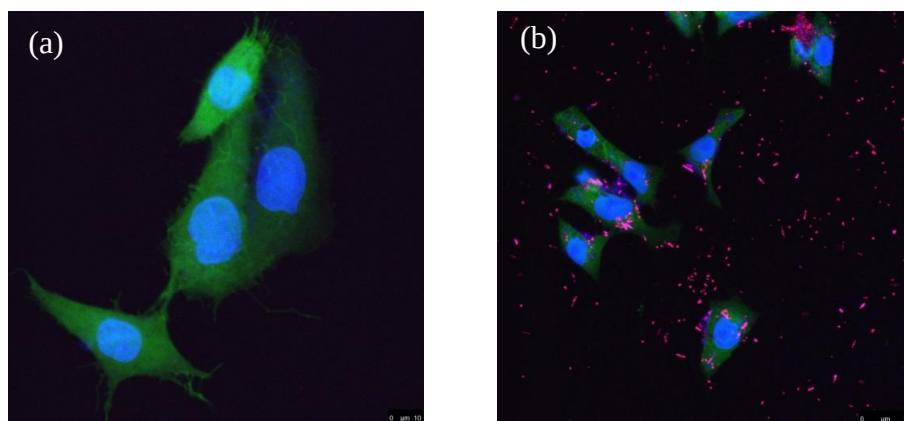


Fig. 3. The confocal microscopic data control of cell culture (a) and cell culture with SiNLGs (b)

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USING NANODIAMOND'S FLUORESCENCE IN BIOAPPLICATIONS

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Abstract. Nanodiamond (ND) is considered as a promising nanomaterial for bio applications. ND surface can be functionalized with molecular and ionic groups and then functionalized with biomolecules of interest. Spectroscopic properties of NDs make them convenient for imaging and biosensing. The surface-attached molecules serve for specific or non-specific interaction with a target, whereas ND spectroscopic signals are used for detection. ND fluorescence, including non-linear effects (multi-photon excitation, energy transfer, etc.), and the methods of increasing the fluorescence intensity are discussed. The mechanisms of ND interaction with biological systems on different levels of organization are studied using ND spectroscopic properties.

The main origin of nanodiamond (ND) fluorescence is color centers in the diamond lattice [1]. Due to defect nature of the fluorescence, it can be excited in a wide range of excitation wavelengths, is stable and does not reveal photobleaching, intrinsic fluorescence is sufficiently intense for detection. The methods of enhancement are discussed and developed also [1, 2]. Together with fluorescence, ND reveals a strong and isolated Raman signal – phonon mode of sp^3 bonded carbon. In this work we discuss applications of ND spectroscopic properties for bio-imaging and drug delivery tracing.

Application of ND like cellular markers, using ND spectroscopic properties, has been proposed and demonstrated successfully [1-3]. Of fundamental importance for bioapplications is the fact that ND surface can be functionalized with a number of molecular or ionic groups which provide chemical or physical attaching of molecules of interest. Anticancer drugs [4], insulin [5], gen [6], etc. delivery as well as imaging of specific targets [7] have been demonstrated with observation of ND conjugates internalization and localization by the cells or tissues.

In this work we discuss the properties of ND fluorescence, including some non-linear effects [8], which can be useful for further development of new methods of bio-imaging and bio-sensing. It has been shown that the ND fluorescence is emitted predominantly by diamond core, but the surface also can play an important role [9]. The effect of surface oxidation on fluorescence spectra shape and lifetimes were demonstrated and discussed in [10]. Analogous effects were observed at hydrogenation of ND surface. The metal-enhanced fluorescence (together with surface enhanced Raman scattering) has been observed for ND during interaction with Ag thin nanostructured films [11]. As the surface modifying and interactions can affect the ND fluorescence, new opportunities open for bio-sensing applications. The effects of attached macromolecules on the ND on the ND fluorescence spectra and lifetimes have been observed. Energy transfer between ND surface, color defects and surface attached fluorescent dye molecules [12] or adsorbed non-fluorescent proteins [13] was studied.

Multiphoton excitation of ND fluorescence is observed for different color centers [14]. The multiphoton excitation for ND biomarkers opens the possibility to use IR for excitation and, accordingly, to decrease destroying laser effects on living objects, to increase light penetration in the sample, and spatial resolution; as well as to avoid overlapping of the signal with autofluorescence of biological sample.

The examples of ND fluorescence applications for bio-imaging and bio-sensing are demonstrated and discussed.

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EFFECT OF NANODIAMONDS ON THE MICRORHEOLOGIC PROPERTIES OF BLOOD AND VASOMOTOR REACTIONS OF ISOLATED VESSELS OF RATS UNDER *IN VITRO* AND *IN VIVO* INCUBATION

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Abstract. Various optical techniques were used to study the interaction of diamond nanoparticles – nanodiamonds (ND) with red blood cells (RBCs) of human and rat blood and vasomotor reactions of isolated vessels of rats under *in vitro* incubation of freshly drawn blood samples and vessels with ND and *in vivo* incubation after intravenous administration of ND into live rats. Measurements conducted *in vitro* showed various concentration-dependent effects of 100 nm ND on the functional properties of RBCs and no effect of the tested ND in concentration of 100 µg/ml on the vessels.

Introduction

Bionanophotonic techniques play an increasingly important role in diagnosis of biological objects of different complexity. With a fast growing potential and extensive applications of nanotechnology in biomedicine and in the environment, the issues of “biotoxicity” and “biosafety” of nanoparticles have become of great significance for the researchers. During the last decade ND attracted great attention due to their intrinsic properties of nontoxicity and biocompatibility. However, possible effects of these nanoparticles on biological structures starting from molecular and cellular levels, including blood components, have not been fully assessed so far. The aim of this work was to study the influence of ND on the microrheologic characteristics of human and rat blood at *in vitro* incubation of blood samples with ND and *in vivo* incubation after intravenous administration of ND into live rats. Also, we analysed the impact of ND on the vasomotor reactions of isolated mesentery vessels of rats under their *in vitro* incubation with ND.

Methods and Results

In our previous work [1], we studied various effects of ND on the components of blood plasma. The adsorption of blood plasma proteins on ND was analysed using UV-visible absorption measurements. We showed, in particular, that the adsorption of blood plasma proteins albumin and γ-globulin on diamond nanoparticles sized ~5 and ~100 nm leads to variations of the FTIR spectra of the proteins corresponding to structural transformations of the adsorbed molecules and, consequently, to a significant decrease in the protein functional activity. We also found that the influence of ~5 nm ND on the protein structure and functions is more significant than that of ~100 nm ND. We also studied the influence of ND on the oxygenation states and microrheological properties of RBC *in vitro* [2].

Measurements were facilitated using laser scanning fluorescence and Raman scattering spectroscopy, dynamic and diffuse light scattering techniques, and laser diffractometry (also known as ektacytometry). Diffuse light scattering from freshly drawn whole blood samples after their incubation with ND was used to study the effect of ND on the kinetics of RBC spontaneous aggregation and shear-induced disaggregation. Diffraction of laser beam on diluted suspensions of RBC before and after incubation with ND was implemented for quantitative measurements of the effect of ND on the ability of RBC to deform when subjected to shear stress in a rotational Couette cell [3]. Optical trapping with laser tweezers was used to study the deformability and interaction of individual RBCs suspended in autologous plasma and/or in solutions of various macromolecules that allowed comparing different models of the mechanisms underlying the RBC aggregation process *in situ*. All measurements were performed *in vitro*. However, *in vitro* incubation of blood samples with ND just mimic the *in vivo* conditions. It does not reproduce possible effects of ND on microcirculation via their interaction with the endothelium of the vessel walls.

So one may judge on the functional value of the effects or mechanisms found *in vitro* only in the case that they are confirmed in the *in vivo* experiments. Here we report on the latest results of our

research, which combines our *in vitro* and *in vivo* expertise in cellular and in whole blood and animal models using state of the art laser methods and instruments. Importantly, we show that the ND in their as-prepared form and in physiological condition neither cause hemolysis nor affect the cell viability. Neither the oxygenation/deoxygenation states are altered when the ND interact with RBC. However, in some cases the ND affect the microrheologic properties of RBC such as their ability to change their shape under shear stress as well spontaneous aggregation and shear-induced disaggregation parameters. This may be due to the ND sticking to RBC membrane as well as due to adsorption of blood plasma proteins on their surfaces, which we recorded by various laser techniques. The effects are particle size, concentration and surface functionalization dependent.

The effect of ND in the concentration of 100 µg/ml on the endothelium of rat microvessels was studied by measuring the contractility of the rat mesentery arteries with average diameter 320 µm after their incubation with ND. Experiments were conducted with the myograph model 410A, Danish Myo Technology. The vessel contractility was tested with methoxetamine in the concentrations from 10^{-7} to 10^{-5} M. The safety of endothelium was tested by adding acetylcholine in the concentration of 10^{-5} M. In all vessels the endothelium was found in nonaffected state. In such experimental conditions we did not observe any effect of ND on the vasomotor reactions of isolated vessels.

Conclusion

These results imply that controlling the blood (micro) rheologic properties is necessary during the ND application for *in vivo* experiments and clinical trials. Different techniques based on laser interaction with particles can be efficiently used for this purpose. We believe that this conclusion is true for all nanoparticles designed for biomedical applications albeit their administration into the organism is to be performed via blood flow. Further *in vivo* experiments are of crucial importance for testing the results obtained in *in vitro* conditions.

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MORPHOLOGICAL ANALYSIS OF THE NANOPARTICLES-LABELED TUMORS AFTER LASER TREATMENT

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Introduction

The majority of works on laser therapy of cancer with nanoparticles show tumor ablation [1-3]. But the most preferable approach for future clinical application is mild controlled hyperthermia [4]. There are some researches demonstrating that the efficiency of laser hyperthermia is higher with the use of gold nanoparticles [5, 6]. However, one of the most important things to understand the efficacy of plasmon resonant nanoparticles is to investigate the character of tumor damage induced by laser therapy with gold nanoparticles.

The aim of the research was to study the morphological changes in the tumors after laser therapy in combination with gold nanoparticles.

Materials and Methods

Nanoparticles. An aqueous colloid of plasmon resonant gold nanobranched (43 µg/ml) coated by polyethyleneglycol (PEG) was explored. The size of the nanoparticles was about 200 nm. The extinction peak was at the wavelength of 850 nm, which is optimal for the optical imaging and laser hyperthermia. Solution of the nanoparticles was administrated intravenously.

Animals. The study was performed on female CBA mice bearing cervical carcinoma. The tumor was transplanted subcutaneously. Animals were divided into three groups: treated by laser with nanoparticles (n=10), treated by laser (n=10), untreated (n=10).

Optical imaging. A noninvasive control of nanoparticle accumulation in the tumor was carried out *in vivo* by the optical coherence tomography (OCT) system designed at the Institute of Applied Physics (IAP RAS, Russia). The OCT system had the following characteristics: a probing radiation wavelength of 900 nm, power 2 mW, spatial resolution 15-20 µm, imaging depth up to 1.5 mm. OCT images were obtained before the nanoparticle administration and afterwards every 30 min for 5 hours. The nanoparticles uptake in tumor was estimated by an increase of the OCT signal intensity.

Laser hyperthermia. The laser treatment was performed in 5 hours after the nanoparticle injection. The tumors were exposed for 20 min to light from an 810 nm diode laser. The power density was 1.2 - 1.5 W/cm². The superficial tumor temperature was controlled to be between 44-45°C by means of a standard IR- thermograph. Tumor size measurements were taken daily for 7 days, and then tumor growth inhibition (TGI) was calculated [7].

Morphological analysis. Histological examination of the tumors was performed 1, 3 and 7 days after the treatment. For histological analysis the animals were sacrificed, tumors were surgically removed and fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin. Four micrometer sections were stained with hematoxylin and eosin (H&E) and examined with light microscopy.

Results

OCT monitoring of nanoparticles accumulation in the tumors

Accumulation of the nanoparticles in tumors provided some optical changes on the OCT-images. The first one was enhanced brightness of the images relative to the control without nanoparticles. The second change was an increase of the signal penetration depth. The time when the optical changes were most pronounced was found to be 4-5 hour after the injection, indicating maximum accumulation of the gold nanoparticles in the tumor tissue [8].

Laser hyperthermia of tumor in combination with gold nanoparticles

Applying of the gold nanoparticles to laser therapy had a few benefits. The most important one was very fast heating. Tumors with nanoparticles reached the peak at 44°C in 3 minutes. It was three times faster in comparison with tumors treated by laser only. To remain the temperature the same for 20 minutes it was necessary to decrease the laser power from 1.2 W to 0.9 W.

It was also demonstrated using IR-thermography that nanoparticles-labeled tumors were heated locally [8].

Tumor growth inhibition

Laser hyperthermia in combination with gold nanoparticles provided an antitumor effect. The maximal value of TGI was 51% on the 7th day after laser treatment in combination with gold nanoparticles. In case of laser therapy of the nanoparticles-free tumors the mean of TGI appeared to be negative on the 7th day, indicating an intensive growth of the tumor.

Morphological analysis

To study the process of cancer cells death after laser therapy with gold nanoparticles histological examination of the tumors was performed 1, 3 and 7 days after treatment. Histopathology revealed that tumor tissue changed in the same manner under the laser treatment with gold nanoparticles and without them. Untreated (control) tumors had a typical dense structure and contained areas of spontaneous necrosis and areas of disconnected round shape cells. Laser treatment provoked an increase of the area of disconnected cells, extension of necrosis and decrease of a typical dense tissue area. At the same time, nuclei became hyperchromic and dark-pink cytoplasm condensed under laser treatment. Importantly, laser treatment in combination with nanoparticles caused more significant changes.

Conclusions

In the present study the efficacy of the gold nanoparticles for laser therapy of tumors was demonstrated. It was shown by optical imaging *in vivo* that the time of maximum nanoparticle accumulation in the tumor was 4-5 hours after intravenous injection. Application of gold nanoparticles for laser hyperthermia led to faster tumor heating with less laser energy and resulted in tumor growth inhibition over 50%. Morphological examination revealed significant decrease of the typical cancer tissue area.

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BRIGHT CIRCULARLY PERMUTED VARIANTS OF FLUORESCENT PROTEIN FUSIONRED

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Abstract. Performance of the currently available red fluorescent proteins is often unsatisfying when expressed as fusion proteins. We have recently reported a new monomeric red fluorescent protein, called FusionRed, which demonstrates both high efficiency in fusions and low toxicity in living cells and tissues. Here we report a number of bright circularly permuted variants of FusionRed as a first step for both single FP-based and FRET-sensors construction. We hope that this finding will provide a base for the construction of new generations of bright red fluorescent sensors with low toxicity and high labeling efficiency.

Multicolour labeling with fluorescent proteins is frequently used to differentially highlight specific structures in living systems. Labeling with fusion proteins is particularly demanding, but is still problematic with the currently available palette of fluorescent proteins that emit in the red range. This is due to unsuitable subcellular localization, protein-induced toxicity and low levels of labeling efficiency. We have recently reported a new monomeric red fluorescent protein, called FusionRed, which demonstrates both high efficiency in fusions and low toxicity in living cells and tissues [1]. Besides its advantages as a fluorescent tag, this protein represents a great base for development of fluorescent sensors, which would inherit all properties of FusionRed.

Table 1. Properties of various permuted variants of red fluorescent protein FusionRed

Permutation point *	Brightness in <i>E.coli</i> , % of FusionRed	Extinction coefficient, M ⁻¹ cm ⁻¹	Quantum yield	Brightness (QY*EC), M ⁻¹ cm ⁻¹	Brightness, % of FusionRed
189-188	133	109 400	0,13	14200	62
169-168	114	81 300	0,14	11400	50
152-151	111	147 800	0,14	20700	90
168-167	105	96 900	0,13	12600	55
167-166	95	105 900	0,16	17000	74
150-151	75	124 300	0,16	19900	87
168-168	63	119 100	0,17	20200	89
167-167	61	116 600	0,17	19800	87
167-168	59	125 200	0,15	18800	82
143-143	17	---	---	---	---
143-144	14	---	---	---	---
144-144	13	---	---	---	---
145-144	8	---	---	---	---
142-141	0	---	---	---	---
142-142	0	---	---	---	---
142-143	0	---	---	---	---
142-144	0	---	---	---	---
143-142	0	---	---	---	---
144-143	0	---	---	---	---

* Permutation point is presented by amino acids position numbers in the sequence of FusionRed. The same numbers mean that the amino acid was kept at both N- and C-ends of the permuted variant.

Here we report a number of bright circularly permuted variants of FusionRed as a first step for both single FP-based and FRET-sensors construction. Some of these permuted variants demonstrate increased brightness as compared to FusionRed itself, when expressed in *E.coli* (Table 1). The brightest variant, with C and N-ends corresponding to the positions 188 and 189 at the parental protein, demonstrates 33% increased brightness. We hope that this finding will provide a base for the construction of new generations of bright red fluorescent sensors with low toxicity and high labeling efficiency.

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LENS LESS HOLOGRAPHIC MICROSCOPY AND SPECKLE PHOTOMETRY IN BIOPHOTONICS

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Abstract. Optical microscopy is well known as an imaging technique with a resolution in the wavelength region using lenses. It is also possible to detect directly interferences of a microscopic object followed by a numerical image reconstruction. Depending on the numerical aperture the same lateral resolution is reached known for optical microscopes. The holographic inline microscopic imaging, its principle, its development steps for miniaturization, the state of the art as well as fields of application are presented.

Principle of digital holographic microscopy

The holographic principle for detection and reconstruction of far field wave fronts was invented by D. Gabor more than 60 years ago. Gabor illuminated a pinhole aperture with a mercury vapor lamp and recorded an interference pattern on a photographic plate. This interference pattern is also called hologram. In digital inline-holographic microscopy the object is illuminated by a partially coherent light source and the hologram is recorded by a digital image sensor (CCD, CMOS). An image of the object is reconstructed numerically with a computer.

Today, the technical effort required for the production of microprocessors needed for the reconstruction of the image is less than the effort required for the manufacturing of a typical microscope lens.

The current status is characterized by an efficient algorithm for lens-free holographic microscopy, a micro coherent illumination system, and a lens-free setup for incident light microscopy.

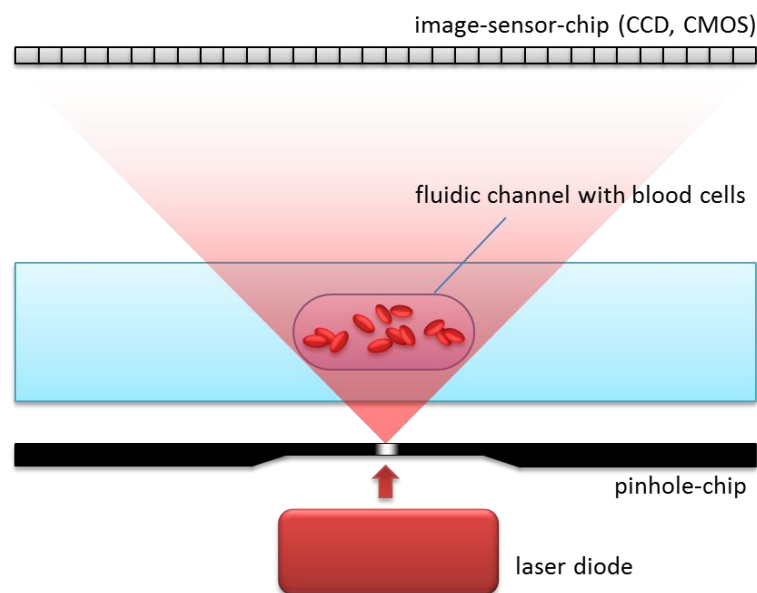
Reconstruction algorithm, setup in reflection mode and micro coherent illumination system

A new efficient reconstruction algorithm allows a spatial resolution corresponding to a numerical aperture of 0.85. The resolution is referred in detail down to the region of 800 nm in case of using red light (661 nm). This approach to microscopy is efficient, and less expensive.

A new patented incident light setup enables a separate lens-free “microscopy unit” which is suitable for macroscopic samples such as skin and food. The incident light setup can be assembled with planar chips. In addition, the known limit of the conventional in-line approach does not apply to the incident light setup. Samples can now completely cover the entire image field.

We adjust the degree of coherence (corresponding length 10 ... 200 μm) for suppressing disturbing interferences.

Such a lens-free microscope can be made with planar chips.



Applications

Application examples are shown. An example is holographic imaging of samples in micro fluidic channels (blood cell diagnosis). As an application the imaging of the flow of human blood cells in a microfluidic channel is presented. Therefore different refractivities along the optical path, e.g. air-glass-water-glass-air, are taken in consideration.

Speckle Photometry

In addition to imaging, this setup can be used for speckle photometry as well. Speckle photometry reveals information about surface structures and their changes under stress. Applications can be found, for example, in medicine (cell structure diagnosis in tissue) or in the characterization of material surfaces in security technology. By a fractal correlation technique the structure of human skin is characterized and various cosmetic properties can be derived (in collaboration with J. Schreiber, Dresden, Germany).

Finally an overview is given about the activities and the trends in the world in the field of lens less microscopy comparing with modern products of so called digital microscopes.

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UPCONVERSION NANOMATERIALS FOR FLUORESCENT BIOIMAGING

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Abstract. Spectroscopic characteristics of highly photochemical stable nanoparticles from complex polycrystalline oxide rare-earth compounds $\text{Gd}_2\text{GeMoO}_8$, $\text{La}_4\text{Gd}_{10}\text{B}_6\text{Ge}_2\text{O}_{34}$, and $\text{Gd}_{11}\text{SiP}_3\text{O}_{26}$, doped with pairs of rare-earth elements $\text{Yb}^{3+}\text{-Er}^{3+}$ and $\text{Yb}^{3+}\text{-Tm}^{3+}$, in which upconversion luminescence can be excited were investigated. The quantum yield and lifetime of upconversion luminescence at 978 nm excitation are determined for individual electronic transitions in VIS region, which is used to optimize the composition of dopants in matrices. The dependences of the upconversion luminescence intensity on the pump power were obtained and analyzed [1].

Background. Highly photochemical stable nanoparticles, in which upconversion luminescence can be excited – the so-called upconversion nanocrystals (UC-NCs) – exhibit widely separated (up to 500 nm) narrow luminescence bands in the VIS region located far from the excitation NIR laser radiation, and thus can be more easily identified compared to organic luminophores and semiconductor nanoparticles. Due to a deep penetration of exciting IR radiation, the absence of parasitic fluorescence of biomolecules and the absence of phototoxicity and photobleaching upon near IR excitation, UC-NCs can be efficiently used as fluorescent probes in biological studies and fluorescence diagnostics [2]. The doping of such nanoparticles with Gd^{3+} ions provides an additional possibility of combining fluorescence visualization with magnetic resonance imaging, which will considerably improve the sensitivity of diagnostics of cancer tumors even at the early stages [3].

Materials and methods. Silicate phosphates $\text{Gd}_{11-x-y}\text{Yb}_x\text{Er}(\text{Tm})_y\text{SiP}_3\text{O}_{26}$ were synthesized by co-precipitation of the initial components hydroxide REE from aqueous solutions by ammonia [4] with followed precursor filtration, drying and heating at $T = 1400^\circ\text{C}$ to obtain the finished product [5]. $\text{Gd}_{2-x-y}\text{Yb}_x\text{Er}(\text{Tm})_y\text{GeMoO}_8$ and $\text{La}_3\text{Gd}_{11-x-y}\text{Yb}_x\text{Er}(\text{Tm})_y\text{B}_6\text{Ge}_2\text{O}_{34}$ were synthesized by the method of solid-phase interaction of corresponding oxides heated to the final synthesis temperature 1250°C [6, 7]. According to the X-ray phase analysis data, the synthesized nanopowders doped with Yb^{3+} , Er^{3+} and Tm^{3+} are single-phase and isostructural to undoped matrices standard.

Upconversion luminescence spectra in the VIS region, which were further used to calculate the absolute quantum yield, were measured with a setup in which a sample under study was placed inside a modified integrating sphere Avantes (Avantes BV, Netherlands). The exciting IR radiation 974 nm was introduced inside the sphere, and scattered radiation and upconversion fluorescence was collected from the sphere with an optical fiber connected with a spectrometer. The instrumental function of the spectrometer was determined by calibrating the setup with the help of lightemitting diodes (LEDs) emitting at different wavelengths. The quantum yield of upconversion luminescence was calculated from the expression: $QY = P_{\text{VIS}}/P_{\text{abs974}}$, where P_{VIS} is the upconversion emitted light power in the range of 400–800 nm, and P_{abs974} is the output power of the 974 nm laser (at pump power densities 0.5 and 2.5 W/cm^2) absorbed by the sample equal to the difference of the scattered radiation power for the referenced sample and the power by the sample under study.

The upconversion luminescence intensity I_{vis} in the VIS spectral region depends on the pump power I_p as $I_{\text{vis}} \propto I_p^n$ where n is the number of IR photons absorbed upon emission of one photon in the VIS region [8]. The slope of the dependence of the upconversion luminescence intensity on the pump power is determined by the competition between relaxation processes and upconversion during the population of excited states of the acceptor.

To study the kinetic characteristics of upconversion luminescence of nanoparticles, we propose a new method using a scanning microscope LSM-710-NLO (Carl Zeiss Group, Jena, Germany). Upconversion luminescence in the VIS region was excited at 974 nm by a tunable laser (780–1080 nm) Chameleon (Coherent Inc., USA) in the non-confocal non-descanned detector (NDD) regime. Because the luminescence lifetime of samples is much longer than the scan time per pixel, luminescence tracks are obtained, which after the subsequent program processing and approximation can be used to determine the rise and decay times of upconversion luminescence.

Results. The upconversion luminescence spectrum of samples containing Er^{3+} exhibits the 480 nm $^2\text{P}_{3/2} \rightarrow ^4\text{I}_{11/2}$, 525 nm $^2\text{H}_{11/2} \rightarrow ^4\text{I}_{15/2}$, 545 nm $^4\text{S}_{3/2} \rightarrow ^4\text{I}_{15/2}$, and 665-nm $^4\text{F}_{9/2} \rightarrow ^4\text{I}_{15/2}$ bands, while the spectrum of samples with Tm^{3+} exhibits the 475 nm $^1\text{G}_4 \rightarrow ^3\text{H}_6$, 650 nm $^1\text{G}_4 \rightarrow ^3\text{F}_4$, 786 nm $^1\text{G}_4 \rightarrow ^3\text{H}_5$, and 800 nm $^3\text{H}_4 \rightarrow ^3\text{H}_6$ bands. The maximum quantum yield of luminescence in the VIS range for samples doped with $\text{Yb}^{3+}\text{-Er}^{3+}$, was 0.97%, 1.09% and 1.36%, for $\text{Gd}_{9.9}\text{Yb}_{0.99}\text{Er}_{0.11}\text{SiP}_3\text{O}_{26}$, $\text{La}_3\text{Gd}_{10.16}\text{Yb}_{0.7}\text{Er}_{0.14}\text{B}_6\text{Ge}_2\text{O}_{34}$ and $\text{Gd}_{1.78}\text{Yb}_{0.14}\text{Er}_{0.08}\text{GeMoO}_8$, respectively; and for samples doped with $\text{Yb}^{3+}\text{-Tm}^{3+}$ without the inclusion of highly effective transitions $^1\text{G}_4 \rightarrow ^3\text{H}_5$ (786 nm), $^3\text{H}_4 \rightarrow ^3\text{H}_6$ (800 nm), was 0.33%, 0.60% and 1.32%, for $\text{Gd}_{9.79}\text{Yb}_{0.66}\text{Tm}_{0.33}\text{SiP}_3\text{O}_{26}$, $\text{La}_3\text{Gd}_{9.74}\text{Yb}_{0.84}\text{Tm}_{0.42}\text{B}_6\text{Ge}_2\text{O}_{34}$, and $\text{Gd}_{1.82}\text{Yb}_{0.14}\text{Tm}_{0.04}\text{GeMoO}_8$, respectively.

To establish the frequency conversion mechanism for radiation from the 974 nm IR laser exciting upconversion luminescence in the 525 nm, 545 nm, and 665 nm bands of Er^{3+} ions and the 475 nm, 650 nm, 786 nm, and 800 nm bands of Tm^{3+} ions, we plotted the dependence of the upconversion luminescence intensity I_{VIS} on the power density I_p of laser radiation in a double logarithmic scale. It can be said that the slope of the upconversion luminescence intensity I_{VIS} from the exciting radiation power density I_p changes practically for all the samples at a power density about 1 W/cm², but if for samples doped with Er^{3+} the dependence increases, then for transition $^1\text{G}_4 \rightarrow ^3\text{H}_6$ Tm^{3+} ion the slope of the curve decreases.

The luminescence tracks of nanopowders with the rapid rise and slow decay intervals corresponding to the $^2\text{H}_{11/2} \rightarrow ^4\text{I}_{15/2}$ and $^4\text{S}_{3/2} \rightarrow ^4\text{I}_{15/2}$ transitions of Er^{3+} for the $\text{Gd}_{11-x-y}\text{Yb}_x\text{Er}_y\text{SiP}_3\text{O}_{26}$, $\text{Gd}_{2-x-y}\text{Yb}_x\text{Er}_y\text{GeMoO}_8$ and $\text{La}_4\text{Gd}_{10-x-y}\text{Yb}_x\text{Er}_y\text{B}_6\text{Ge}_2\text{O}_{34}$ confirm the two-photon nature of the energy transfer process. The luminescence lifetime of Er^{3+} decreases with increasing concentration of Yb^{3+} for all types of matrices. For example, for $\text{La}_3\text{Gd}_{10.16}\text{Yb}_{0.7}\text{Er}_{0.14}\text{B}_6\text{Ge}_2\text{O}_{34}$ a relatively high intensity of transitions ($^2\text{H}_{11/2}$, $^4\text{S}_{3/2}$) $\rightarrow ^4\text{I}_{15/2}$ and a long lifetime of luminescence of 100 μs was observed, whereas for $\text{La}_3\text{Gd}_{9.32}\text{Yb}_{1.26}\text{Er}_{0.42}\text{B}_6\text{Ge}_2\text{O}_{34}$ the luminescence lifetime was 26 μs . The same tendency was observed for the lifetime of luminescence transition of Er^{3+} $^4\text{F}_{9/2} \rightarrow ^4\text{I}_{15/2}$. For samples with $\text{Yb}^{3+}/\text{Tm}^{3+}$ there is a more obvious tendency of reduction in the luminescence lifetime of Tm^{3+} $^1\text{G}_4 \rightarrow ^3\text{H}_6$ with increasing concentrations for all types of matrices, except $\text{La}_3\text{Gd}_{11}\text{B}_6\text{Ge}_2\text{O}_{34}$. However, the luminescence lifetime remains at the same high level of 100 μs . At comparable $\text{Yb}^{3+}\text{-Er}^{3+}$ and $\text{Yb}^{3+}\text{-Tm}^{3+}$ dopant concentrations, the upconversion luminescence lifetime for the $\text{La}_3\text{Gd}_{11}\text{B}_6\text{Ge}_2\text{O}_{34}$ matrix is 2.5–15 times higher than for $\text{Gd}_2\text{GeMoO}_8$ and $\text{Gd}_{11}\text{SiP}_3\text{O}_{26}$.

Conclusion. Based on the experimental results obtained, REI pair $\text{Yb}^{3+}\text{-Er}^{3+}$ is good for energy transfer in the green and/or red part of the spectrum as well as for the FD and can be used for a further energy transfer to the photosensitizers at photodynamic therapy (PDT). REI pair $\text{Yb}^{3+}\text{-Tm}^{3+}$ transform the infrared radiation in the blue region of the spectrum, which is also suitable for FD and PDT and additional intensive energy conversion in NIR will allow deep tissue imaging. The upconversion NPs long luminescence lifetime is another advantageous property; it can be useful for the time-resolved bioimaging.

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A BRIGHT MONOMERIC GREEN FLUORESCENT PROTEIN WITH A FLUORESCENCE LIFETIME OF 5.0 ns

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Abstract. Cyan fluorescent proteins (CFP) with tryptophan66-based chromophore are widely used for live cell imaging. In contrast to green and red fluorescent proteins (FPs), no charged states of the CFP chromophore have been described. We have recently developed the first fluorescent protein with anionic tryptophan-based chromophore and a record-holding fluorescence lifetime of 5.2 ns. Here we report its enhanced variant named WasCFP2 with fluorescence lifetime of 5.0 ns, improved brightness, maturation rate and stability of the anionic form of chromophore. We expect WasCFP2 to be a useful tag for fluorescence lifetime imaging microscopy (FLIM) as well as a promising base for development of sensors for FRET-FLIM imaging.

Cyan fluorescent proteins (CFP) with tryptophan66-based chromophore are widely used for live cell imaging. In contrast to green and red fluorescent proteins, no charged states of the CFP chromophore have been described. Recently we studied synthetic CFP chromophore and found that its indole group can be deprotonated rather easily (pK_a 12.4). We then reproduced this effect in the CFP mCerulean by placing basic amino acids in the chromophore microenvironment. As a result, a green-emitting variant with an anionic chromophore and key substitution Val61Lys was obtained. We named it WasCFP (W in anionic state CFP) [1].

At physiological conditions WasCFP exists in equilibrium between two forms: with neutral cyan-emitting and with anionic green-emitting chromophores. This equilibrium is very sensitive to changes in pH, temperature and urea concentration.

Here we report the results of the extensive random mutagenesis of WasCFP aimed at enhancing its brightness, maturation rate and stability of the anionic form of chromophore. The final mutant named WasCFP2 (17 amino acid substitutions apart from mCerulean) is a very bright monomeric fast-maturing green fluorescent protein with a fluorescence lifetime of 5.0 ns. This is the highest fluorescence lifetime value among all known FPs (with an exception for its parental protein, WasCFP) and about twice-higher value than that of an average green FP.

This work provides the first evidence strongly suggesting that tryptophan-based chromophores in fluorescent proteins can exist in a charged state. Since WasCFP2 possesses such an exceptionally long fluorescence lifetime, we expect it to be a useful tag for fluorescence lifetime imaging microscopy (FLIM) as well as a promising base for development of sensors for FRET-FLIM imaging.

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RAMAN SPECTROSCOPY – A POWERFUL APPROACH TOWARDS LABEL FREE BIOMEDICAL DIAGNOSTIC

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Abstract. Raman based techniques are very powerful biomedical diagnostics tools because the Raman effect provides high specificity (i.e. molecular fingerprint information). Here, we will present modern trends in Raman microspectroscopy for biomedical applications. It will be shown how linear and nonlinear Raman microscopy methods have been successfully applied for a labelfree morphochemical characterization of complex tissue samples potentially even in-vivo for an early diagnosis of diseases like e.g. cancer.

During the last years Raman based microspectroscopy has been recognized as an extremely powerful tool for bioanalytical and biomedical applications because the method provides molecular fingerprint information of the molecular structure and biochemical composition of cells and tissues without external markers [1, 2]. Diseases and other pathological anomalies are accompanied by changes in these properties.

Here, we briefly describe some of our latest results concerning the application of linear and nonlinear Raman microspectroscopy to characterize a broad range of different tissue sections (biopsy specimens) for biomedical diagnosis.

We will start with showing that the processing of the chemically specific tissue Raman-maps via mathematical approaches for a spectral analysis and classification enables an objective evaluation of the tissue samples for an early disease diagnosis like e.g. cancer [3] or inflammatory bowel disease [4]. Besides these ex-vivo tissues Raman studies (i.e. Raman pathology) first steps towards in-vivo Raman spectroscopy that is Raman endospectroscopy will be presented. By doing so novel Raman fiber probes for an intraoperative monitoring of the arteriosclerotic plaque in living rabbits will be presented [5].

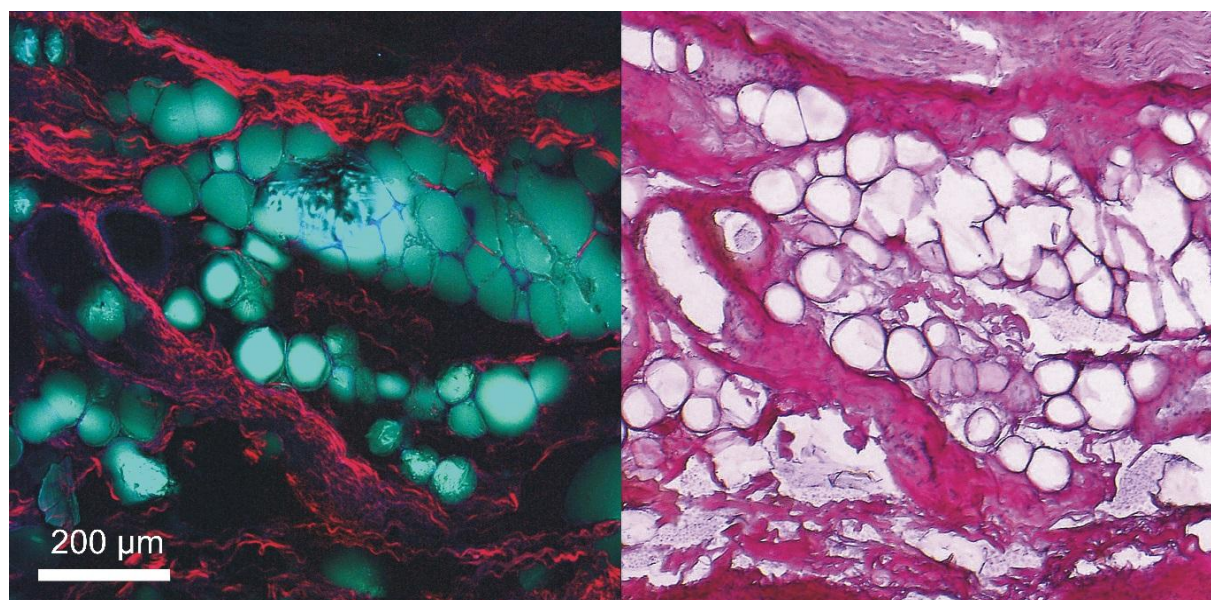


Fig. 1. Right: non-linear multimodal image of a human atherosclerotic plaque deposition at the inner wall of the aorta (blue: CARS (proteins); green: CARS (lipids); red: TPF/SHG); left: histopathological staining

The low Raman scattering cross section results in long acquisition times limiting the recording of Raman images of large tissue areas and thus, clinical applications. The acquisition times can be reduced by utilizing non-linear Raman approaches like CARS (coherent anti-Stokes Raman scattering)

and allows recording Raman images of single characteristic Raman bands in real time. It will be shown, that the joint use of linear Raman microspectroscopy and CARS microscopy allows for complementary characterization of the type and chemical composition of the tissue samples [6]. While linear Raman microspectroscopy is used to obtain the information on critical Raman marker bands at selected spatial position within the sample, CARS microscopy is focusing on fast image generation using the previously defined Raman marker bands. In order to improve the diagnostic result CARS microscopy can be easily combined with second harmonic generation (SHG) and two-photon fluorescence (TPF) microscopy. SHG and TPF highlight morphological / structural features by displaying collagen structures (SHG) and the spatial distribution of autofluorophores like e.g. NAD(P)H, flavines, elastine, etc. Overall we will present the development of a compact CARS/SHG/TPF multimodal nonlinear microscope in combination with novel fiber laser sources for use in clinics [7]. The diagnostics potential of this compact multimodal microscope as compared to conventional histopathological images has been demonstrated for the examples of cancer [8-11] and atherosclerosis [7] (see Figure 1). These examples show the great potential of multimodal imaging to complement established clinical pathological diagnostic tools.

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NONLINEAR OPTICAL CORRELATION SPECTROSCOPY

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Abstract. We present a novel concept for optical spectroscopy called nonlinear correlation spectroscopy (NLCS). NLCS analyses coherent field fluctuations of the second and third harmonic light generated by diffusing nanoparticles. Particles based on noncentrosymmetric nonlinear materials such as KNbO₃ show a strong second as well as third harmonic response. The method and the theory are introduced and experimental NLCS results in fetal calf serum are presented showing the promising selectivity of this technique for measurement in complex biological environments.

Introduction

Current single-molecule methods are limited by their signal-to-noise ratio (SNR) at both short and long observation times. For fast processes, their SNR is determined by the lifetime and quantum yield of fluorescent labels and by the detection efficiency of the instrument. On the other hand, it is also difficult to measure slow processes with fluorescence methods because fluorophores hardly withstand long exposure times but tend to photobleach. Photobleaching typically restricts the total observation time to a few seconds, which complicates characterizing slow processes. In order to overcome these shortcomings, novel techniques based on nonlinear optics such as Raman correlation spectroscopy (RCS) [1], coherent anti-Stokes Raman scattering correlation spectroscopy (CARSCS) [2], and sum-frequency scattering [3] have been developed. A novel approach named nonlinear correlation spectroscopy (NLCS) is presented here, which is also free from photobleaching and has the potential to become a valuable tool for spectroscopic measurements.

Experimental Setup

We use a chromium-activated forsterite laser (Cr:forsterite) emitting fs-pulsed light at a central wavelength of $\lambda_0 = 1250$ nm. Consequently both second harmonic (SH, at 625 nm) as well as TH (417 nm) light can be detected easily with standard optics. Such an approach is advantageous with respect to Ti:Sapphire laser-based systems, where the resulting TH appears in the deep-ultraviolet (DUV) at ≈ 260 nm and is therefore absorbed by most standard glasses employed in microscope objectives. Figure 1A shows a schematic drawing of our setup.

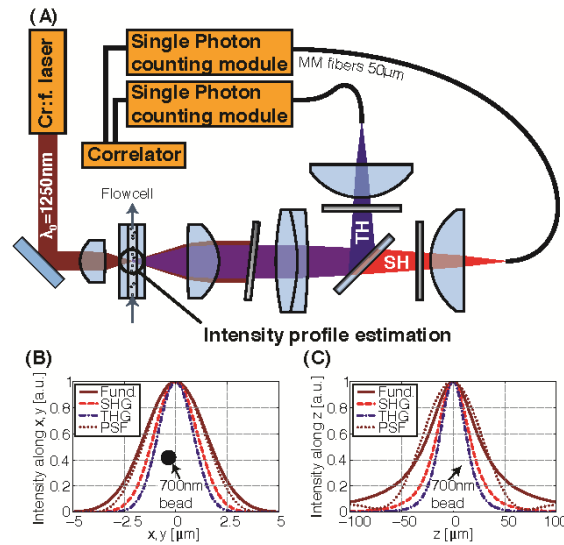


Fig. 1. (A) Nonlinear correlation spectroscopy (NLCS) setup. (B) and (C) Intensity profile in xy and z as predicted by Zemax calculation (PSF) as well as by Gaussian beam approximation for the fundamental light intensity, as well as the SHG and THG profiles

Results

Measuring in complex environments such as blood serum is a challenging task and requires high sensitivity in order to overcome background contributions. For instance, the autofluorescence typically prohibits sensitive fluorescence measurements in blood serum. With our instrument, we performed NLCS in fetal calf serum (FC serum). We employed serum that had been inactivated by heating at 56°C during 30 min and subsequently filtered with a pore size of 0.22 μm . Although a SH field can be theoretically generated at any interface including PS NPs surface) the yield of this process is quite low, and in our experimental conditions we were not able to detect any SH nonlinear signal. On the contrary, the use of noncentrosymmetric NPs can greatly enhance the nonlinear generation efficiency, because in this case the process originates from within the particle. To observe a SH signal, we used KNbO_3 NPs (size before coating 120 nm) prepared by mechanical grinding, followed by selected centrifugation and deposition. This approach presents the advantages of high signal stability (i.e., no photobleaching), wavelength flexibility, and coherent SH and TH emission. Not surprisingly the serum contains structures emitting TH light (Figure 2A). Moreover, we could detect some rare and weak SH emission. This FC serum background showed a stable TH autocorrelation. After adding the KNbO_3 suspension, we observed strong higher harmonic generation both in the third and the second harmonic channel and obtained a stable and robust crosscorrelation between the two channels (Figure 2B–D). Figure 2C shows the KNbO_3 autocorrelation curves in both channels. As expected, the SH channel showed a larger focal volume than the TH as confirmed both by the fitted number of particles inside the volume ($N_{\text{SH}} = 0.035$ and $N_{\text{TH}} = 0.018$) as well as by the fitted axial transit times ($\tau_{\text{fz,SH}} = 13$ ms and $\tau_{\text{fz,TH}} = 9.1$ ms).

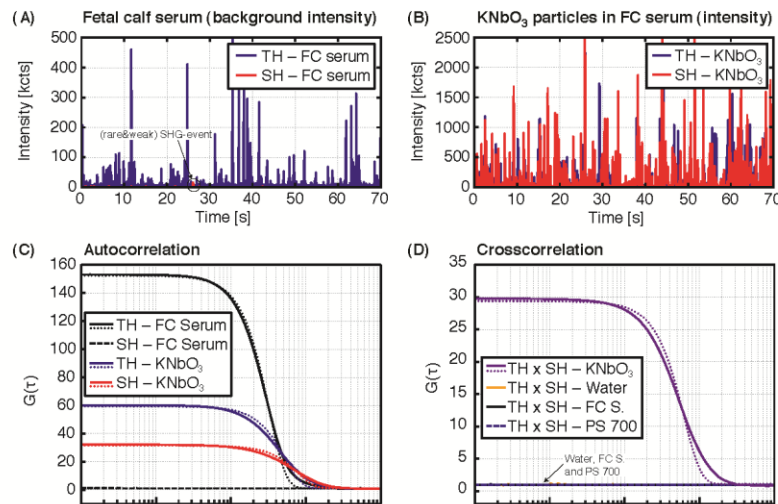


Fig. 2. NLCS measurement in fetal calf (FC) serum

Summary

In conclusion, we have introduced NLCS as a novel spectroscopic method with high sensitivity in complex environments for long-term observation of nonbleaching NPs with a high potential for more applications. These advantages enable sensing and tracking of single NPs as well as long-term imaging inside confined volumes, such as cells, where there is in general no quick renewal of labels. The nondestructive generation of SH and TH by nanoparticles overcomes the photobleaching limitations of fluorescence based methods. And multiharmonic detection combined with the crosscorrelation measurements provides unprecedented selectivity in physiological fluids or other complex environments. This approach can be further refined by the use of polarization resolved detection or by resolving the emission anisotropy (e.g., forward vs backward emission).

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STUDY OF NOVEL PHOTSENSITIZERS BASED ON THE CYANOPORPHYRAZINE CHROMOPHORS INCORPORATED INTO BIOCOMPATIBLE POLYMERIC BRUSH NANOPARTICLES

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Abstract. The goal of our study was to test the photobiological properties of a potential photosensitizing agent based on the watersoluble polymer brush nanoparticles (PBNps) incorporating fluorescent tetra(4-fluorophenyl)tetracyanoporphyrzine free base (Pz). Pz is expected to be an efficient novel tetrapyrrolic photosensitizer for fluorescence diagnostics and photodynamic therapy due to its optical and biomedical characteristics. We demonstrated that PBNps-Pz strongly fluoresces in 630-670 nm region in aqueous solution. PBNps-Pz selective accumulation in tumor were observed; their intracellular locations were presented by nuclear and perinuclear regions; cell investigations confirmed a high photodynamic activity of the nanoparticles.

Materials and methods

Novel fluorescent porphyrzine free base (tetra(4-fluorophenyl)tetracyanoporphyrzine) was prepared (Fig. 1).

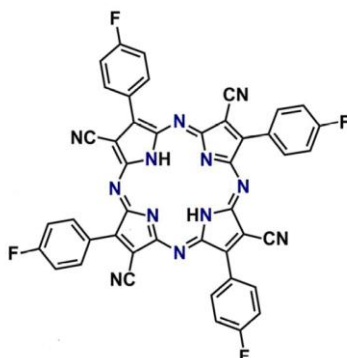


Fig. 1. Structures of tetra(4- fluorophenyl)tetracyanoporphyrzine free base (Pz)

The stable biocompatible form of Pz was obtained by its incorporation into water-soluble polymer brush nanoparticles (PBNps) based on hydrolyzed polyimide-*graft*- polymethacrylic acid. Absorption and fluorescence spectra of Pz and PBNps-Pz were investigated in various media. The influence of biological liquids, such as blood serum and aqueous albumin solution, on the PBNps-Pz fluorescent properties were also studied.

For *in vitro* research of cellular uptake of the PBNps-Pz we used A431 cell line (human epithelial carcinoma). Cells were incubated with the PBNps-Pz for 60 minutes. Intracellular localization of the PBNps-Pz was studied by confocal laser scanning microscopy. Also the dynamics of accumulation of the PBNps-Pz in cells was investigated.

Light and dark toxicity of PBNps-Pz was investigated *in vitro*. The half maximal inhibitory concentration (IC₅₀) was determined for both cases.

Whole-body imaging experiment was carried out using IVIS Spectrum (Caliper, USA) with epiluminescence imaging function. The experiments were performed on female Balb/c mice bearing metastatic colorectal carcinoma. To investigate the PBNps-Pz pharmacokinetics, the mice were imaged *in vivo* in 15 min and 1-4, 6, and 48 hrs after administration. The image obtained before injection was used as a control one. Quantification of the fluorescence in the tumor area provided an opportunity to define tumor uptake and retention kinetics. For verification of the PBNps-Pz accumulation in tumor tissue and investigation of its accumulation in internal organs, fluorescence was analyzed by standard methods — confocal microscopy and fluorescence spectroscopy *ex vivo*.

Results

The Pz THF-solution and PBNps-Pz demonstrated light absorption with maxima at, respectively, 610 nm and 560 nm. Strong fluorescence was shown in a wide range (up to 50 nm) with central peak located at 640-660 nm. Both emission and excitation peaks were shifted to the shorter wavelengths for the PBNps-Pz.

A large enhancement of red emission of the PBNps-Pz in serum and albumin solution was detected. This effect is supposed to result from the PBNps-Pz bindings to proteins.

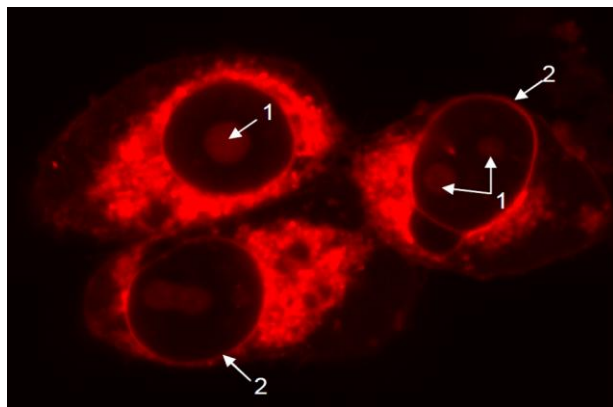


Fig. 2. PBNps-Pz localization in cells. 1 – nuclei, 2 – nuclear membrane

In the cell culture experiments the PBNps-Pz were shown to be internalized and accumulated in the tumor cells on the nuclear membrane and nucleus. The accumulation of PBNps-Pz in nuclear and perinuclear regions has a very significant value in PDT, because nuclear membranes are most vulnerable to photodamage.

The IC₅₀ dose for both light and dark toxicity was shown to be, respectively, 14.8 μ M and much higher than 50 μ M. It confirms the efficacy of synthesized PBNps-Pz as a potential agent for photodynamic therapy.

Experiments on mice bearing metastatic colorectal carcinoma demonstrated rather suitable pharmacokinetics of the PBNps-Pz. Two hours after intravenous injection of the PBNps-Pz in 15 mg/kg dose, they were accumulated in the tumor tissue and retained there up to 7 hours. After 24 hrs complete elimination of PBNps-Pz from the animal body was observed. The experiment shows high tumor selectivity of the potential photosensitizer. The whole-body-imaging results agree with the data of standard *ex vivo* methods.

Conclusions and perspectives

In general, the PBNps-Pz are of interest as a photosensitizer for fluorescence diagnostics and photodynamic therapy due to their optical and biomedical characteristics. PBNps-Pz showed strong fluorescence in 630-670 nm region in aqueous solution; accumulated selectively in tumor; intracellular locations are presented by nuclear and perinuclear regions. The PBNps-Pz are shown to have no dark toxicity, but high photodynamic activity. Hereafter we plan to study photodynamic activity of the PBNps-Pz *in vivo*.

Acknowledgements

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IN VIVO STUDY OF GENETICALLY ENCODED PHOTOTOXIC PROTEINS FOR TUMOR THERAPY

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Abstract. We present here the results of investigation of phototoxic effects of genetically encoded photosensitizers KillerRed and miniSOG on HeLa tumors in mice. The phototoxic proteins targeted to mitochondria and/or nuclei were selected based on *in vitro* data on their phototoxicity and ability of the cell lines to form the tumor. Three treatment regimens were tested. Photobleaching of the proteins was estimated *in vivo*. Pathomorphological study of the KillerRed-expressing tumors revealed essential abnormalities in the tissue structure. Laser treatment of miniSOG-expressing tumors did not induce any signs of phototoxicity.

At present, two fluorescent proteins are known to have phototoxic properties – GFP-like protein KillerRed and flavoprotein miniSOG. KillerRed was engineered in 2006 in Lukyanov's group [1]. Its pronounced phototoxicity has been shown on cancer cells *in vitro* [2, 3]. It has been established that the rate of toxicity and mechanism of the cell death is determined by localization of the phototoxic protein to a large degree. Very recently, light-induced tumor tissue damages have been demonstrated *in vivo* on the HeLa tumor expressing KillerRed [4]. It is proposed that KillerRed works as type I photosensitizer through the generation of superoxide anion radical [5]. FMN-binding protein miniSOG was first reported in 2011 by X. Shu and co-authors as a genetically encoded tag for correlated light and electron microscopy [6]. Later on, its phototoxicity was used for photoablation of neurons in *C. elegans* [7]. In contrast to KillerRed, miniSOG is type II photosensitizer producing singlet oxygen.

The phototoxic proteins are of great interest for photodynamic therapy (PDT) of cancer as genetically encoded photosensitizers. Encoding of the photosensitizer in the cell genome can solve the problems of non-specific accumulation in healthy tissues, re-distribution within the tumor and precise delivery to the particular cell compartments.

This study is aimed at the development of the PDT regimens for cancer treatment with genetically encoded photosensitizers KillerRed and miniSOG on animal tumor models.

The study was performed on immunodeficient *nude* mice, females, with subcutaneously (s.c.) inoculated HeLa (human cervical carcinoma) tumors. Stably transfected cell lines expressing fluorescent proteins KillerRed and miniSOG in different cell compartments and non-expressing control were used. During the selection of the optimal localization of the proteins and treatment regimen fluorescence whole-body imaging was carried out using IVIS-Spectrum system (Caliper Life Science, USA) or home-built setup for epi-luminescence imaging (IAP RAS, Russia). Photobleaching of the proteins was estimated *in vivo*. To confirm the protein expression and photobleaching on the cellular level confocal fluorescence microscopy *ex vivo* was performed on LSM 510 META23 system (Carl Zeiss, Germany). In order to prevent thermal effects, the temperature of the tumor surface was controlled. Three treatment regimens with variations in light dose, number of irradiation procedures and tumor stage at the beginning of PDT were tested. After the treatment, early tumor response was investigated by pathomorphological analysis.

For the purpose of the choice the optimal localization of the phototoxic protein KillerRed in cancer cells, five cell lines were evaluated for the ability to form the tumor node. It was revealed that HeLa cells expressing KillerRed on the cytoplasmic membrane do not generate the solid tumor efficiently. The tumors expressing KillerRed in the lysosomes had very low fluorescence intensity, presumably due to lysis of the protein by the lysosomal enzymes. The cells with KillerRed of other locations (mitochondrial - KillerRed-mito, nuclear fused with histone H2B - KillerRed-H2B, and double KillerRed-mito-H2B) displayed 100% inoculation, and the tumors were easily identified by KillerRed fluorescence since day 10th after s.c. cell injection. Based on this data and the previous *in vitro* results KillerRed-mito, KillerRed-mito-H2B, miniSOG-mito, miniSOG-H2B were selected.

Irradiation of the KillerRed-expressing tumors with laser at the wavelength of 594 nm at a power density of 150 mW/cm² over 20 or 30 min caused photobleaching of KillerRed, which appeared in

fluorescence intensity decrease by 25% or 31 % correspondingly (fig. 1, upper row). In case of tumors expressing miniSOG, we observed photobleaching both endogenous fluorophors in skin and miniSOG in tumor after irradiation at 473 nm. However, weak transparency of the tissues for blue light did not allow fluorescence imaging of the tumors *in vivo* (fig. 1, lower row). In the extracted tumors the fluorescence fell by a factor of 3.5 for miniSOG-H2B and 10 for miniSOG-mito in comparison with non-treated tumors.

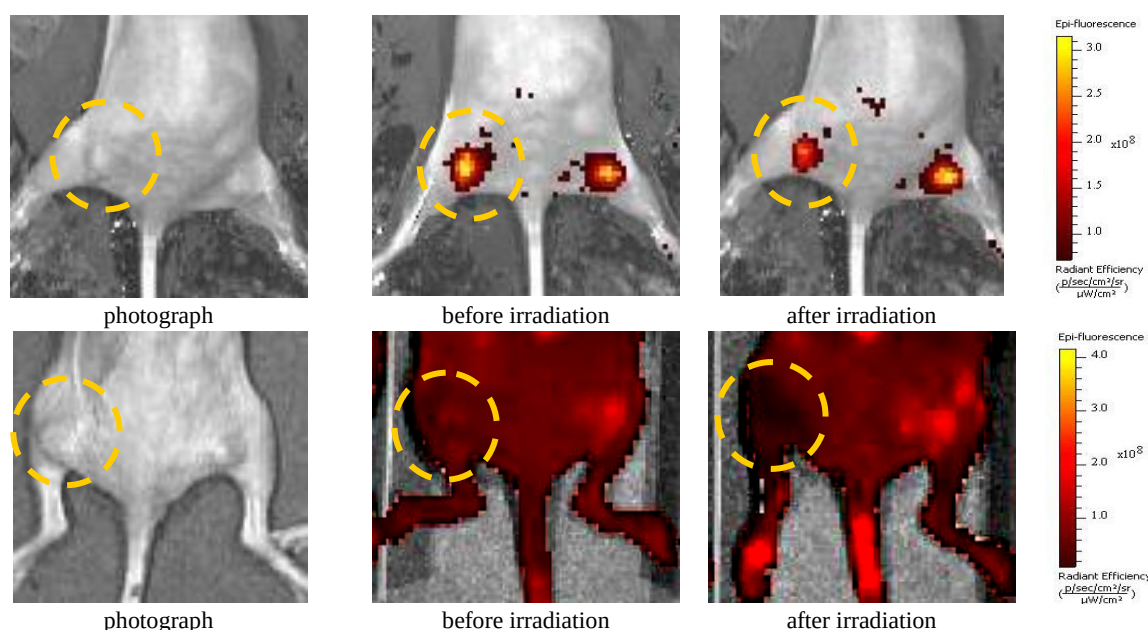


Fig. 1. *In vivo* fluorescence imaging of HeLa tumors expressing KillerRed-H2B (upper row) or miniSOG-mito (lower row). Tumors were inoculated on both legs. Irradiated tumor is shown by dashed circle. For KillerRed ex. 570 nm, em. 620 nm. For miniSOG ex. 465 nm, em. 520 nm

Pathomorphological study of the KillerRed-expressing tumors 24 h after the last laser treatment revealed essential abnormalities in the tissue structure. The cancer cells had strongly vacuolized cytoplasm, swollen nuclei, broken cariolemma and plasma membrane. An increased number of apoptotic cells in the treated tumors expressing KillerRed-mito-H2B was found. When the stronger regimen was applied (150 mW/cm², 30 min, 7 times since day 5th of the tumor growth), the morphological changes were more pronounced.

Laser treatment of miniSOG-expressing tumors did not induce any signs of phototoxicity. This can be explained by FMN deficiency in poor vascularized HeLa tumors preventing recovery of miniSOG fluorescence and phototoxic action.

The results show a therapeutic potential of KillerRed for PDT. Despite the high phototoxicity of miniSOG *in vitro*, its testing on HeLa tumor xenografts displayed the loss of photodynamic activity. Our future efforts will be on optimization of the therapeutic regimen, involving other tumor models, and exploring the mechanism of PDT with genetically encoded photosensitizers.

Acknowledgements

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INNOVATIVE METAL AND SEMICONDUCTOR NANOSTRUCTURES FOR (BIO)-PHOTONIC APPLICATIONS

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Abstract. Plasmonic core-shell and dendrite-like nanostructures have been realized by applying different formation techniques. The SER(R)S activities of such nanostructures were investigated. Significant Raman signal enhancement was demonstrated in numerical simulations as well as experimentally in measurements of SER(R)S activity of a highly dilute model dye molecules. The optical phenomena like enhanced Raman scattering, low reflection, high optical absorption and room temperature light emission were observed in chemical “Black Silicon”. The metallic and “black silicon” nanostructures with extra high active surface and phenomenon optical properties are opening us the novel possibility to observe in situ the biological, chemical, biochemical processes.

The formation of metal and silicon nanostructures mediated by thin film technologies or wet-chemical approach has successfully been demonstrated and will be discussed in details in our presentation. The noble metals are very well suited to serve as active substrates for Surface Enhanced (resonant) Raman Spectroscopy (SER(R)S). SER(R)S is a technique that was developed to detect extremely small quantities of molecules, for example, biological molecules by determining their characteristic Raman signal, i.e. their characteristic vibrational modes. The high sensitivity of SER(R)S has a mainly electromagnetic origin described as an effective energy transfer of the incident light which is concentrated to the nanoscale onto the metal nanoparticles. These couplings of photons to the corresponding free electron gas eigen-modes are known as surface plasmons that can be used to excite vibrational modes in the adsorbents. Particular strong intensity enhancements are expected at positions where adsorbents stay in close vicinity (few nanometers apart) to the so-called hot spots (points of strong field enhancement in between nanoparticle dimer). Realizing core-shell metallic nanoparticles with different metals in core and shell, and different shapes/dimensions of core/shell it is possible to tune material absorption or extinction spectra to the vibrational modes of the adsorbents of interest.

Our suggested SER(R)S active surfaces are composed of silicon nanowires etched from [1] the silicon wafer using AgNO_3/HF etch solutions or grown by chemical vapor deposition technique. These nanowires account for a large surface area. Platinum nanoparticles were realized in controlled process by ALD using a commercially available atomic layer deposition reactor (PICOSUN, Finland). The formation and morphology of platinum nano-islands on silicon nanowire surfaces was realized and will be discussed in details [2]. Pt is however hardly plasmonically active. We therefore use the Pt-islands only as a nanoscale vehicle for selective autometallographic deposition of thin, plasmonically active noble metal layers atop. Auto-metallographic deposition of silver (Ag) or gold (Au) layers on the Pt nanoparticles was realized and plasmonic activity of the core//shell Pt//Ag or Pt//Au material was demonstrated in numerical simulations as well as experimentally in measurements of SER(R)S activity of a highly dilute model dye molecule. The morphology and structure of the core-shell nanoparticles on SiNWs surfaces was investigated by scanning and transmission electron microscopies. The optimum geometry for maximum field and Raman signal enhancement in metal Pt/(Ag, Au) core/shell nanoparticles on silicon nanowires will be discussed essentially based on the finite element modeling.

In the second part of the presentation we will show that dendrite like silver nanostructures grown by wet-chemical approach of silver, can produce a significant enhancement of Raman scattered signals [3]. Signal enhancement for a few or even just single silver dendrite is demonstrated by analyzing the enhanced Raman signature standard dye molecules, bio-molecules or drugs.

Semiconductor nanowires have been attracting large interest as a very promising approach toward post CMOS nano-electronics, for photonics, for energy, and life science, as well as for fundamental materials science and physics studies. In the present study we investigate the growth process, structure, optical properties and bio-application possibilities of silicon nanowires obtained by top-down or metal assisted chemical etching (MACE) of crystalline silicon [1]. The MACE technique is

based on selective electrochemical etching using catalytic silver and can give ordered and densely packed arrays of high aspect-ratio single crystalline silicon nanowires (SiNWs) with uniform crystallographic orientations. The optical phenomena like Raman scattering, low reflection and high optical absorption of such nanostructures will be discussed [4, 5]. The strong shortening of light-matter interaction time in SiNW arrays is obviously interesting for various photonic applications as non-linear optical conversion of the light frequency and duration of short laser pulses. Another field of application of the observed effect of light localization is sensing of molecules incorporated or/and absorbed into SiNW array. The detection of enhanced molecular response at characteristic vibration frequencies can be realized by means of the IR reflectance/transmittance and Raman spectroscopy. The nature and dynamic of room temperature photoluminescence in silicon nanowires will be discussed in detail [6]. First results indicate that SiNWs could penetrate into the cells via an endocytosis mechanism and can be used as powerful luminescent labels for living cells.

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PATHOMORPHOLOGICAL CHANGES IN THE TUMORS INDUCED BY PDT WITH GENETICALLY ENCODED PHOTSENSITIZERS

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Genetically encoded photosensitizers are a promising optogenetic instrument for light-induced production of reactive oxygen species in desired locations within cells *in vitro* or whole body *in vivo*. Only two such photosensitizers are currently known, GFP-like protein KillerRed and FMN-binding protein miniSOG. The study of phototoxic effects of KillerRed and miniSOG in experimental tumors enables understanding the mechanisms of action of genetically-encoded photosensitizers and choosing the directions for photodynamic therapy (PDT) development.

The goal of the study was to investigate pathomorphological effects of the proteins KillerRed and miniSOG on animal tumors after photodynamic therapy in different treatment modes.

The study was carried out on immunodeficient *nude* mice with subcutaneously implanted tumor HeLa (human cervical cancer). The tumor cells stably expressed genetically-encoded photosensitizer KillerRed in chromatin and mitochondria (KillerRed-mito-H2B) or in chromatin alone (KillerRed-H2B), and miniSOG in different localizations, namely, mitochondria (miniSOG-mito) or chromatin (H2B-miniSOG). Control tumors were HeLa tumors without the proteins.

Two treatment regimens with a total fluence of 270 J/cm² (strong regimen) and 180 J/cm² (soft regimen) were tested. The irradiance was 150 mW/cm². The light was delivered through an optical fiber and irradiated the tumor surface over a 7 mm-diameter beam spot. The animals were divided into the following groups: “no treatment, no photosensitizer”, “no treatment, photosensitizer”, “treatment, no photosensitizer” and “treatment, photosensitizer”. There were 8 groups of animals with miniSOG and 8 groups of animals with KillerRed, each group included 4-5 animals.

The tumors expressing miniSOG were treated with a blue laser at the wavelength of 473 nm (MGL, CNI, P.R. China) at a fluence of 270 J/cm² (150 mW/cm² for 30 min) daily for 10 days from day 10th of the tumor growth.

The tumors with KillerRed were irradiated with yellow laser (MGL, CNI, P.R. China) at 593 nm wavelength at two regimens - 270 J/cm², 150 mW/cm², 30 min every day for 7 days starting from the 5th day of the tumor growth for KillerRed-mito-H2B and 180 J/cm², 150 mW/cm², 20 min three times every other day starting from the 13th day of the tumor growth for KillerRed-H2B.

For histological examination, 24 h after the last treatment the animals were sacrificed, tumors were surgically removed and fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin. Four micrometer sections were stained with hematoxylin and eosin (H&E) and examined with light microscopy. The cancer cells in H&E stained slides of each tumor were counted in 8–18 randomly selected microscopic fields of 0.01 mm² at x400 magnification. Percentage of the typical (unaltered) cells, altered cells, and mitotic figures was measured. The altered cells included cells with dystrophic changes and cells with apoptosis hallmarks. Mean±SD values were used for the expression of the data. Statistical differences in percentage of estimated cells between groups were determined by unpaired Student's t-test. Statistical significance was defined at the level of $P < 0.05$ (two-tailed).

Surprisingly, no phototoxicity for tumor cells *in vivo* was observed for either miniSOG-mito or H2B-miniSOG on subcutaneous HeLa tumors. The illuminated and non-illuminated tumors grew similarly, and had no significant histological differences between them. The tumors had a compact dense structure and consisted of clusters of large polymorphic cancer cells separated by **connective** tissue fibers thin-walled small blood vessels. The cancer cells had large round or oval nuclei containing fine dispersed chromatin. Lightly basophilic cytoplasm gathered around the nucleus as a thin ring. Vascularization of the tumors was poor.

Histological examination showed that untreated non-expressing KillerRed HeLa tumors had a typical dense tissue structure and consisted of large polymorphic cells tightly packed together. The tumor cells formed complexes surrounded by thin layers of the connective tissue. The unaltered (without any morphological changes) cancer cells amounted to 82-84 % of the total number of cancer

cells in the field of view (Tables 1 and 2). At the same time, the cell population in the tumors was heterogeneous. This heterogeneity was indicated by the different extent of dystrophic cellular changes. In particular, the cells with vacuolated cytoplasm, swollen or small irregular hyperchromic nuclei were also observed. The proportion of such cells totaled 7-9%. The apoptotic cells counted about 7%.

Table 1. Summary of histological analysis of HeLa tumors with or without KillerRed-mito-H2B after laser treatment at fluence of 270 J/cm² (M±SD)

	No treatment		Treatment	
	No KillerRed	KillerRed	No KillerRed	KillerRed
Unaltered tumor cells, %	84.3±0.9	82.4±4.5	74.3±1.9*	21.1±3.3*
Altered tumor cells, %	14.7±1.1	16.2±4.0	24.1±1.7*	77.7±2.6*
Dystrophic changes, %	6.9±0.8	9.9±3.1	18.8±0.9*	63.7±4.9*
Apoptosis hallmarks, %	7.8±0.5	6.3±2.1	5.3±1.5 [#]	14.0±3.4*
Mitosis hallmarks, %	1±0.2	1.3±0.7	1.6±0.5	0.8±0.7

Each number represents an average over four animals in each group. *, P < 0.05, compared with all other groups; [#], P < 0.05, compared with groups “no-KillerRed, no-treatment” and “KillerRed, treatment” (Student’s *t* test).

Table 2. Summary of histological analysis of HeLa tumors with or without KillerRed-H2B after laser treatment at fluence of 180 J/cm² (M±SD)

	No treatment		Treatment	
	No Killer Red	KillerRed	No KillerRed	KillerRed
Unaltered tumor cells, %	82.8±3.3	77.8±1.2	76.0±0.3 [#]	36.1±9.8*
Altered tumor cells, %	16.3±2.9	21.7±1.0 [#]	22.6±0.3 [#]	63.7±9.8*
Dystrophic changes, %	9.3±0.5	15.5±0.3 [#]	16.0±2.6 [#]	58.8±9.9*
Apoptosis hallmarks, %	7.0±2.9	6.2±0.7	6.7±2.3	4.8±2.4
Mitosis hallmarks, %	0.8±0.6	0.4±0.2	0.7±0.5	0.2±0.1

Each number represents an average over four animals in each group. *, P < 0.05, compared with all other groups; [#] - P < 0.05, compared with groups “no-KillerRed, no-treatment” (Student’s *t* test)

In the treated tumors without KillerRed a slight increase in the portion of dystrophically changed cells up to 18.8% in strong regimen (Table 1) and 16.0% in soft regimen was found (Table 2). The cell structure aberration observed was mainly cytoplasm vacuolization.

In contrast, extensive morphological changes were found in the treated tumors expressing KillerRed-mito-H2B. Most cells had strongly vacuolized cytoplasm, to the extent of plasma membrane destruction in some cells. In a fraction of the treated cells, nuclei, round or irregular, were enlarged due to swelling, and their cariolemma was broken. The percentage of the altered tumor cells increased to 77.7% in strong regimen (Table 1). In soft regimen applied for the tumors expressing KillerRed-H2B the percentage was 63.7% (Table 2).

In conclusion, the tumors expressing KillerRed after PDT were found to have signs of tissue destruction. Along with moderate, reversible dystrophic changes such as enlargement and reduction in cell size and cytoplasm vacuolization, irreversible changes were revealed in the majority of cells, namely, damage of cellular and nuclear membrane integrity. The performed study showed for the first time the possibility of tumor photodamage using phototoxic protein KillerRed as genetically encoded photosensitizer.

Acknowledgments

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DEVELOPMENT OF MICROFLUIDIC BIOREACTORS FOR SYNTHETIC BIOLOGY

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Abstract. Microfluidic bioreactors were pioneered over a decade ago as tools for early bioprocess development. The dramatic reduction in operating volume, the integration with optical sensors for the real-time monitoring of analytes, and the parallelization capability as a characteristic of microfluidic technology make microfluidic bioreactors an attractive tool for bioprocess development and synthetic biology. Whilst most microfluidic bioreactors were developed to support batch operation mode, use of microfluidic flow concepts to perform continuous culture experiments is of particular relevance to Synthetic Biology: continuous culture performed in chemostat mode provide a steady-state environment for the cells, and thus allow the rigorous characterization of *de novo* engineered cells.

A number of screening methods to synthesize biological parts exist. Native and *de novo* parts can be inserted into host strains and proteins expressed. After screening and cell modification, the expression levels are typically assessed in simple batch culture experiments. These assessments are often performed in simple flask experiments with little regard to the growth conditions the cells are under, and are often performed with different analytical experiments. This then prevents comparability between the obtained results which limits their usefulness for later. We are seeking to validate selected parts of the complex regulatory circuits of bacteria using a microfluidic approach. In particular, we are developing a technology to culture gram-positive bacteria under well-controlled growth conditions in parallelised, chemostat-operated microfluidic bioreactors.

Microbioreactors have been developed that enable fermentation of prokaryotic and eukaryotic microbes, yet most of them can only operated in batch culture mode [1]. Additionally, only few have been developed with gram-positive bacteria [2, 3]. Operating with tiny volumes of a few microliters and below limits the ability to take samples and thus most off- or at-line analyses. However, fermentation variables relevant to finding optimum growth conditions, and relevant to scale-up to larger reactors, such as oxygen, can be measured using commercially available optical sensors. Advantages of these sensors, which operate using the quenching of fluorescence by the analyte, include the non-invasive character of optical detection and the fact that they are not consuming the measured analyte [4]. The combination of real-time monitoring of variables and the multiplexing of the microbioreactors present an opportunity to analyse cellular behavior with statistical rigor. [4]

Here, we present an instrumented microfluidic chemostat which is being developed as part of a European Research project in Synthetic Biology (SYNMOD) to determine optimum growth conditions for bacteria. *S. carnosus*, a gram-positive bacterium with a well-characterised genome and of industrial relevance, is used in this project as a chassis for lantibiotic producing genes. Of interest are to find growth conditions that provide highest level of gene product expression as a marker of lantibiotic production. To this end, we developed a microfluidic bioreactor with active stirring and independent control of multiple fluid flow, such as for the introduction of medium, inoculant, acid/base, and an inducer. To achieve the required dilution rates in these small vessels for chemostat operation, all these liquids must be introduced at a very low flow rate. To monitor optical density as an indirect measure of biomass, and to detect fluorescence, an opto-electronic measurement set-up was developed alongside with the development of the reactor. This will result in an instrumented system capable of tightly controlling the environment of the cells and relate the fluorescent gene product expression to different growth conditions, test space for scaled-up fermentations in order to achieve the best yield of both biomass and product.

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PHOTOLUMINESCENT SILICON-BASED NANOPARTICLES AND NANOWIRES FOR BIOMEDICAL APPLICATIONS

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Abstract. Silicon-based nanomaterials are biocompatible, biodegradable and possess efficient photoluminescence. In the present work Si-based nanoparticles and nanowires prepared by different chemical, electrochemical and laser-assisted methods are examined for applications as fluorescent labels and therapeutic agents.

Silicon (Si) nanostructures (nanocrystals, nanoparticles, porous layer, nanowires) are known to be biocompatible and biodegradable [1]. Combined with unique optical properties [2], Si nanoparticles (NPs) look very promising for bioimaging, drug vectoring, photodynamic and ultrasonic therapy [3-5]. Luminescent porous Si NPs can be produced by electrochemical etching of c-Si in hydrofluoric acid solutions [1, 2]. Laser ablation in gaseous and liquid environment emerged as a "green" physical alternative to the conventional chemical or electrochemical methods [6,7]. The ablation in clean aqueous environment (e.g., de-ionized water) can be used to form colloidal Si NP solutions [8]. Ultra-short laser pulses were used to produce stable solutions of pure low-size-dispersed, size-tunable NPs with remarkable fluorescence and singlet oxygen releases [9], which make them important candidates for applications in cancer theranostics. Currently Si nanowires (NWs) formed by metal (Ag)-assisted chemical etching (MACE) [10,11] are of great interest because of their potential applications in various fields as electronics, optoelectronics, photonics, photovoltaics, bio- and chemical sensors. In particular, Si NWs exhibit a strong optical absorption and rather low reflectance in the visible spectral range [11] as well as the room temperature photoluminescence (PL) [12].

In our work Si and SiC NPs were prepared by electrochemical etching of bulk c-Si and 3C-SiC in HF-based solution followed by mechanical grinding of the obtained porous materials [6,7]. Si NWs with diameter of 10-100 nm were prepared by MACE of p-type double-side polished (100)-oriented c-Si wafers with specific resistivity of 1-10 $\Omega\cdot\text{cm}$. The length of Si NWs was controlled in the range from 0.1 to 10 μm by MACE duration. The etching was done at room temperature. The formed Si NW arrays were additionally immersed in concentrated nitric acid to remove residual Ag nanoparticles. Finally the samples were rinsed in de-ionized water and dried in air at room temperature. The obtained layers were transformed into powders and aqueous suspensions by mechanical grinding. The samples were studied by using transmission electron microscopy (TEM), dynamic light scattering and optical spectroscopy techniques. PL properties were investigated by using both conventional spectroscopic equipment and confocal fluorescent microscopy. Fig.1 shows typical TEM images of the obtained samples, which illustrate their nanostructure. The Si NPs and NWs are found to consist of Si nanocrystals with minimal sizes of 1-5 nm.

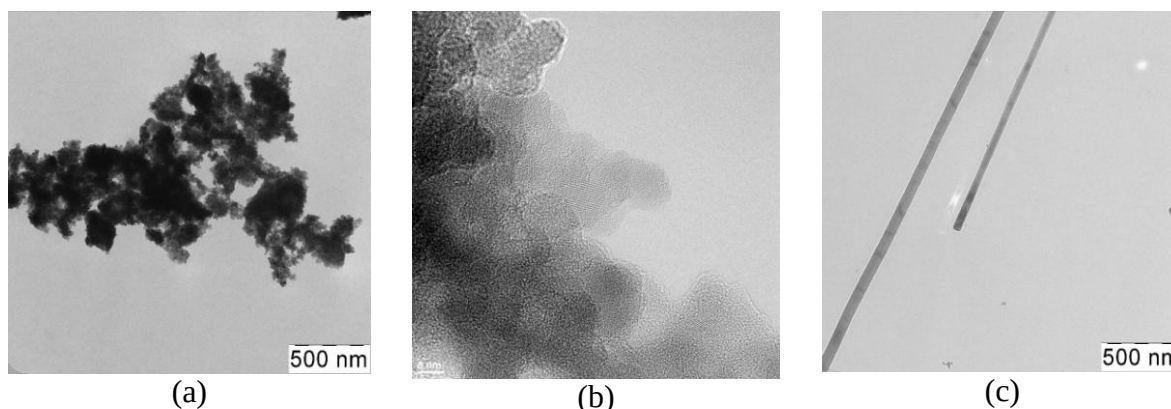


Fig. 1. TEM images of the investigated samples of Si NPs obtained by electrochemical etching (a), laser ablation (b) and NWs prepared by MACE (c)

PL spectra of Si NPs and NWs exhibit maximum varying from 1.2 to 1.8 eV, depending on the conditions of preparation and storage. The PL spectra of 3C-SiC NPs suspended in water consist of a broad band with maximum at 2.2-2.5 eV, depending on the NP size and excitation energies. The PL properties of NPs and NWs are interpreted as a result of the radiative recombination of excitons confined in small nanocrystals – quantum dots (QDs), which consist of the NPs and NWs. In oxygen ambient the PL intensity of Si QDs is found to decrease strongly in comparison with that in vacuum (or in oxygen-free water). The PL quenching in oxygen ambient is maximal at 1.63 eV due to the photosensitization of singlet oxygen generation [5]. In vitro experiments demonstrated that photoexcited Si NPs suppressed the proliferation of cancer cells. Besides the photochemical reaction of Si NPs they could be used as sonosensitizers of local ultrasound-induced hyperthermia and cavitation to destroy cancer cells and tumors in vitro and in vivo, respectively.

For fluorescent bioimaging the aqueous suspensions of NPs and NWs were added to cancer cells in vitro. Figure 2 shows typical fluorescent images of the cells in the presence of Si NPs and NWs. The incorporation of NPs and NWs inside the living cells was monitored over several hours. Under the optical excitation, the NPs marked by red color are rather bright in order to be distinguished from the autofluorescence background of cells. An analysis of the depth profile of the NP fluorescence signal shows that both Si and SiC NPs are localized inside the cells, while Si NWs are located mainly on the cell membranes. Finally, the obtained results demonstrate that Si-based nanomaterials are promising for biomedical applications as diagnostics, drug delivery and PDT. The highly luminescent, stable, and biocompatible NPs of Si and SiC with no protective shells can be applied for fluorescence imaging.

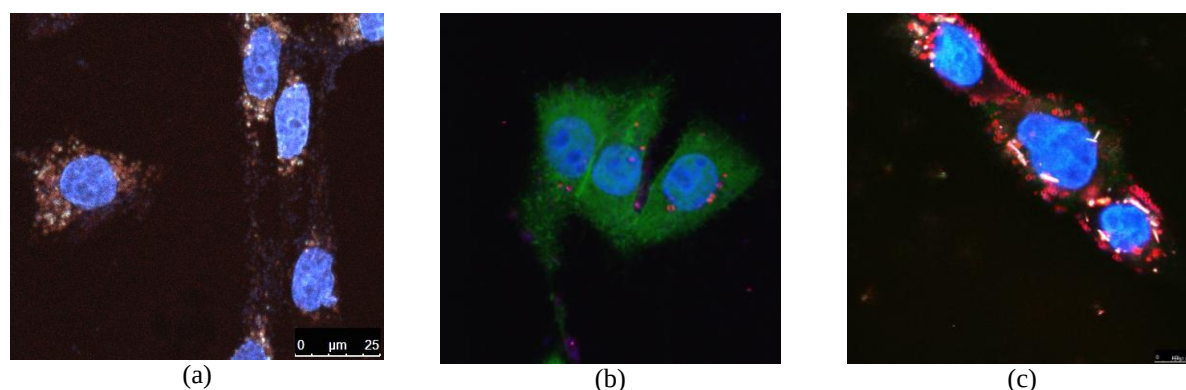


Fig. 2. Fluorescent images of cancer cells with Si NPs obtained by electrochemical etching (a), laser ablation (b), as well Si NWs (c). The Si QD emission and cell nuclei are colored by red and blue, respectively

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MULTI-STABILITY IN COUPLED NOISY REPRESSILATORS

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Abstract. We investigate an experimentally feasible synthetic genetic network consisting of phase repulsively coupled repressilators as a prototypical multi-stable system with coexisting stable attractors with different features. We perform stochastic Gillespie simulations to determine and classify the dynamical structure of the system and the stability of the different regimes in the presence of the intrinsic noise in the genetic system. We compare the stochastic results with deterministic simulations and make use of bifurcation analysis to improve the robustness against the intrinsic genetic noise. In particular we are interested in the stability of the inhomogeneous solutions, which we associate with artificial cell differentiation in isogenetic systems.

Introduction

An important goal of bioengineering is the design and construction of integrated biological circuits that are capable of performing elaborate functions in a cellular context [1]. Synthetic genetic networks which operate almost independently from the rest of cellular machinery and hence provide us with approach a test system of reduced complexity. Such synthetic genetic networks allow studying complicated interaction of gene expression in more precise way. Recent decade manifested itself in pioneering construction of genetic switches, oscillators or logical networks. Genetic oscillators provide the individual cell with a clock controlling vital functions including the cell cycle. The repressilator is a prototype of a synthetic genetic clock built by three genes and the protein product of each gene represses the expression of another in a cyclic manner [2]. It can be constructed experimentally and produce near harmonic oscillations in protein levels. The ability of cell-to-cell communication of living systems [3] is a requisite to ensure an appropriate and robust global cellular response of an organism in a noisy environment.

The noisy repressilator with phase repulsive coupling

In particular, the coupled repressilator is a prototype due to its simplicity yet rather complex dynamics. The basic model consists of a set of coupled differential equations for each cell, which provide very rich and multi-stable dynamics due to phase repulsive coupling [4]. Depending on the cell density and the initial conditions, the system expresses an oscillatory regime, inhomogeneous limit cycle, inhomogeneous steady state and homogenous steady state. Both inhomogeneous states are of particular interest since they can be seen as artificial cell differentiation in isogenetic populations.

Microbiological systems are intrinsically noisy and both natural genetic networks and synthetic genetic networks must cope with the genetic noise evoked from the low copy number of the reactants [5]. In that context, it is important to test if in particular the inhomogeneous solutions persist within a noisy environment. Intrinsic noise can be simulated in the coupled repressilator model by applying the Gillespie algorithm. Taking the parameter set of the deterministic model and applying Gillespie to that model yields very noisy results due to the low copy number of reactants (Fig. 1). To overcome the relative high intrinsic noise level we apply two strategies [6]. First we reduce the intrinsic noise by increasing the number of plasmids within each cell and increase the number of reactants in each cell leading to less intrinsic noise. Secondly, bifurcation analysis shows that the dynamical regimes are close together and the intrinsic noise pushes the system randomly and very frequent in different co-existing state. With help of the bifurcation analysis we try to increase the distance between the dynamical regimes increase the basins of attraction and stabilize the regimes of interest. The bifurcation analysis gives inside the skeleton of the possible dynamical regimes, its stability range and the distance between them. On the one hand multi-stability can be a mechanism to allow a system or organism to make decisions and change the behavior in some instances but on the other hand multi-stability in a noisy system can lead to an erratic behavior with large jumps between the dynamical regimes. The discussed coupled repressilator as a multi-stable system shows both sides and is an interesting test system to study the trade off between stability and noise reduction on one hand and flexibility and variability due to multi-stability and noise induced jumps on the other hand.

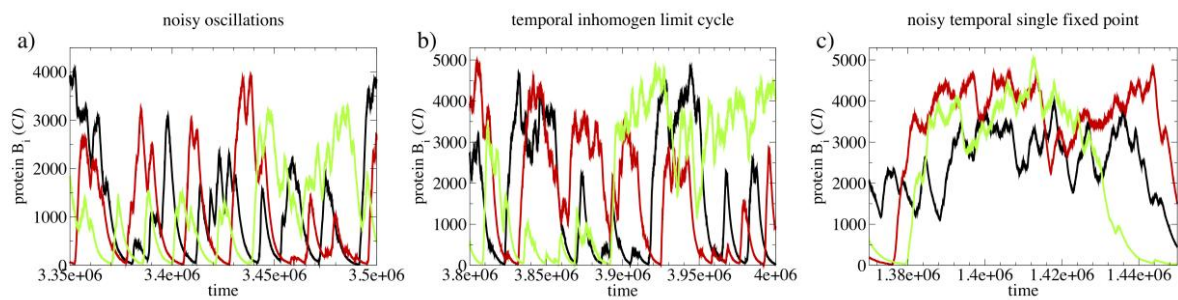


Fig. 1. Stochastic simulation of three coupled repressilators. Each color shows the dynamics of the CI protein in different cell. a) The three cells oscillate under the influence of phase repulsive coupling. b) The genetically identical cells behave differently under the same environmental condition and express an inhomogeneous dynamics. c) The three cells show noisy fluctuations around a common fixed point

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AUTOPHAGY INDUCED BY FEMTOSECOND LASER IN HeLa CELLS

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Autophagy, a homeostatic pathway by which cells break down cellular components under certain conditions, is critical in many types of physiological processes such as immunity and metabolism [1]. Great attention has been attracted to researches of autophagy and significant progresses made in immune diseases, cancers and aging. It can be induced by couples of specific biochemical stimulations, including starvation, energy depletion, immune signals and virus/bacterial toxins [2]. Isolated membrane (also called phagophore) in cells will be formed to engulf certain cytoplasmic materials and the content then degraded by autophagolysosome. Interestingly, in this study it was found that autophagy can be induced in HeLa cells by non-invasive optical stimulation without any physical or biochemical contacts. With ultrahigh peak power and ultrashort pulse duration, femtosecond lasers have shown extraordinary advantages in cell researches [3, 4]. In our investigation, autophagy is found in HeLa cells only after a short exposure of the femtosecond laser without any biochemical involvement. What's more, mitochondrial morphology also changes to some extent by the mean of coordinated fission. By fluorescent co-localization microscopy, the autophagosome does not co-exist with those fragmental mitochondria. Our finding potentially presents a novel regulation of autophagy mechanism.

During the course of autophagy, microtubule-associated protein light chain 3 (LC3), will migrate to concentrate on the autophagosome as a marker of autophagy event [5]. It is thus tagged with green fluorescent protein (GFP) for confocal microscopy. Single HeLa cell was selected randomly for fs-laser (Ti:sapphire) stimulation. After a short exposure on the endoplasmic reticulum (ER), it was found that the dispersed GFP-LC3 partly turned into puncta, symbolized the occurrence of autophagy (Fig. 1). More interestingly, autophagy events were spontaneously observed in surrounding cells, it seemed that the autophagy-activated signals propagated from the targeted cell to its neighbors. As a positive control, cells were cultured in the condition of starvation in EBSS (Earle's balanced salts), autophagy was found. Conversely, there were no changes for a long time in the normal cell medium.

Our previous results indicated that fs laser could induce the release of Ca^{2+} in the ER, decrease of the mitochondrial membrane potential (MMP) and release of reactive oxygen species (ROS) [6]. This ER stress may induce autophagy and the mitochondria may supply isolated membrane meanwhile. In this regard, mitochondrial dynamics during autophagy were studied. After the fs-laser exposure, mitochondrial fission was found (Fig. 2a). During this morphological change, it can be suspected that isolated membrane might be supplied and the fragmental mitochondria might be engulfed for degrading. However, after laser treatment, coincident parts of the GFP-LC3 puncta and the mitochondria (Fig. 2b) can be hardly found, which suggests a minor relativity of the mitochondria membrane and the autophagosome induced by femtosecond laser.

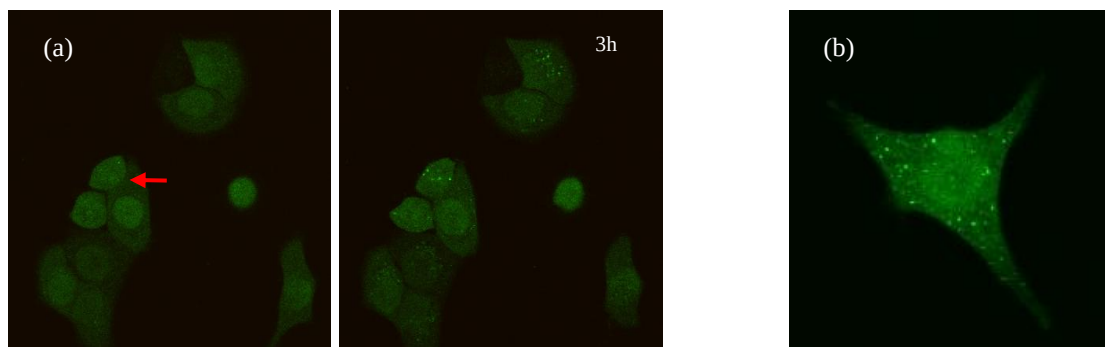


Fig. 1. a) Autophagy induced by femtosecond laser. b) EBSS (Earle's balanced salts) treatment for 2 hours

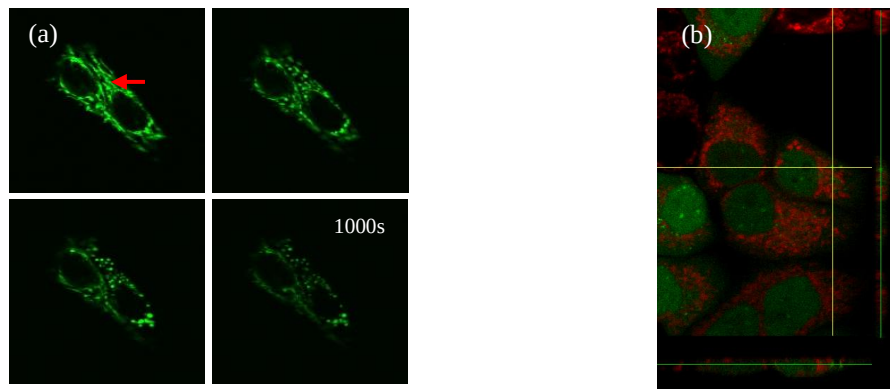


Fig. 2. a) Femtosecond laser induced mitochondria deformation. Mitochondria were labeled by p33-GFP.
b) Co-localization fluorescent microscopy of MitoTracker dyed mitochondria (red) and the autophagosome

In conclusion, it is found that autophagy can be induced by short-time exposure of femtosecond laser without any physical contact or biochemical stimulation. The optical induced mitochondrial fission might not involve in autophagy. Our findings suggest that there may be potential unknown mechanisms of autophagy induced by femtosecond laser.

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EFFECT OF STOCHASTICITY ON CLASSIFYING GENETIC NETWORKS

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Abstract. Relatively simple systems such as cells are able to perform tasks like decision making and learning by utilizing their genetic regulatory frameworks. The biological systems in place to perform such tasks are hugely complex and inherently noisy due to stochastic nature of chemical reactions. In this paper we will consider examples of two such intelligence mechanisms in a biological setting and simulate them numerically, analyzing their behaviour and reactions to noise. We will show that, surprisingly, the correct amount of noise in these systems can have an optimizing effect in these intelligence tasks.

Introduction

Cells make their decisions and choose their fate by superimposing the pattern of extracellular signalling with the intracellular state given by locations and concentrations of chemicals inside the cell. This process is regulated by the program encoded in the cellular genome. Understanding the principles of this programming and, especially, how this program is executed is a key problem of modern biology, for control of this program will provide an insight into new biotechnologies and medical treatments. The 2012 Nobel Prize in Physiology of Medicine was awarded to J.B. Gordon and S. Yamanaka for their discovery that already differentiated stem cells can be reprogrammed to a pluripotent stem cell state [2, 3]. On the other hand, the last decade was marked by the explosion-like progress in the field of synthetic biology. Synthetic biology aims at creating functional devices on the basis of standardized biological building blocks. The advantage of synthetic genetic networks is that they usually function independent of the rest of cellular machinery and, hence, have reduced complexity. As a result, one can analyse not only the topology of the genetic network, but also its dynamics. Rapid progress on synthetic biology in the last decade provided us with different engineered circuits, from toggle switches to complicated logical circuits, with a huge possible area of biological and medical applications [1].

Recent studies, however, have shown that intelligence in biological systems can be implemented not only at the level of intercellular communication as in neural networks, but on intracellular scale. It was shown that neural network can be built on the basis of chemical reactions, if a reaction mechanism has neuron-like properties [6]. In these works linked chains of chemical reactions could act as Turing machines or neural networks [7]. D. Bray has demonstrated that a cellular receptor can be considered as a perceptron whose weights have been learned via genetic evolution [8], showing formally that protein molecules may work as computational elements in living cells [9].

Despite formal proof-of-the-principle experimental work has fallen short to fully implement intracellular intelligence. However, L. Qian et al. have experimentally shown that neural network computations, in particular, a Hopfield associative memory, can be implemented with DNA gate architecture and DNA strand displacement cascades [10]. Hence, before neuron-based brains evolved, complex biomolecular circuits provided individual cells with “intelligent” behaviour required for survival [10]. The same has happened with associative learning: Gandhi et al. have formally shown that also associative learning can be performed in biomolecular networks [11], and in 2008 Saigusa et al. have shown that amoebae can anticipate periodic events [12]. This effect has been explained by the onset and sustaining of intracellular periodic oscillations, without learning or perceptron properties, and it has been discussed that this kind of cellular memory hints at the origins of intelligence [13]. However, in 2008 Rowe et al. have suggested a formal scheme of the single cell genetic circuit which can associatively learn within the cellular life [14]. The same team has investigated with positive result using the real genomic interconnections whether the genome of the bacterium *E. Coli* could work as a liquid state machine learning associatively how to respond to a wide range of environmental inputs [15]. These findings are important also for understanding brain functioning because they show that much simpler organisms and human brains have common aspects in cellular composition and molecular architecture [16].

Methods

Our aim was to analyse the effect of genetic noise on cellular intelligence. In order to check how stochasticity, intrinsically present in any process of gene expression, affects intracellular intelligence, we have considered two different intracellular learning genetic networks. The first one is based on the principle of standard perceptron, developed by Frank Rosenblatt, and the second one works as an associative perceptron mimicking the “Pavlov dog” classical conditioning.

Results

In summary, we have found that in genetic linear classifiers the combination of a spoiled classification threshold and input noise can result in an improvement of accuracy over either of these situations in isolation given the correct circumstances. By modelling this system using probability distributions we calculated both the optimal noise intensity to give the highest value of classification accuracy over all. Considering associative genetic perceptrons, we have found that the genetic associative learning network exhibits highly sensitive switching behaviour, but by considering stochastic concentration trajectories we were able to demonstrate an elongation of the systems memory with increased noise.

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FLIM-FRET OF CASPASE-3 ACTIVATION IN VIVO USING GENETICALLY ENCODED BIOSENSORS

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Abstract. Here we studied the possibility of detecting caspase-3 activity, a key enzyme of apoptosis, in the cell and organism by FRET-FLIM method and factors affecting the analysis of the distribution of the lifetimes. This method allowed not only discriminating apoptotic cells in cell population but also estimating the ratio of bound and unbound donors of biosensor based on FRET-pair of TagRFP as a donor and KFP as an acceptor and excluding the autofluorescence influence.

Introduction

Visualization and quantification of enzymatic activity in a cell is an important task of modern bioimaging. The FLIM-FRET method based on imaging the distribution of lifetimes of the fluorophore (FLIM) and fluorescence resonance energy transfer (FRET) provides a high spatial (nanometer) and temporal resolution (ns) signal, which allows tracing molecular events. Caspase-3 is a key enzyme of programmed cell death apoptosis. When using the fluorescent genetically encoded FRET-biosensors it is possible to monitor activation of caspase-3 *in vivo* detecting the change of distribution of their averaged life-time. Our work is concerned with the study of caspase-3 activation in cells and in living organisms by FLIM-FRET.

Materials and Methods

The fluorescent substrate for caspase-3 TagRFP-23-KFP (TR23K) on the base of pair red fluorescent proteins was developed at our lab earlier [1]. Lentiviral particles containing TR23K and TagRFP were obtained from Evrogen (Russia) and used for transfection of human lung adenocarcinoma A549, human larynx carcinoma Hep-2 and ductal breast carcinoma HBL-100. Different apoptotic agents were used for treating cells *in vitro*. Transduced cells (1×10^6 in 100 μ L) were inoculated subcutaneously to *nude* mice in order to obtain xenograft tumor models expressing the above mentioned constructions. Antitumor drugs were administered with 5-8 days of tumor growth in different schemes. FLIM-FRET of cells was obtained on the time-resolved fluorescent confocal microscopy system Microtime 200. FLIM-FRET of tumor xenografts was obtained on the DCS-120 confocal scanning FLIM systems.

Results and Discussion

The biosensor TR23K is based on a pair of two fluorescent proteins TagRFP and KFP linked by peptide fragment of 23 a.a. This linker contains caspase-3 specific fragment DEVD. Specific cleavage by active caspase-3 leads to the change in fluorescent signal which can be measured. Previous study of TR23K showed the presence of FRET between TagRFP and KFP. Analysis of the distribution of the lifetime in living cells allowed discriminating apoptotic cells from intact within cell population [2].

The conditions of effective cleavage of caspase-3 specific DEVD fragment in transduced cells were found. Narrow life-time distribution of 1.8-2.1 ns (intact biosensor TR23K) in transduced cells shifted to the bimodal distribution with life-time of 1.8-2.1 ns and 2.4-2.6 ns typical for free TagRFP after induction of apoptosis. It was shown that changes in the distribution of cells in lifetime due to some antitumor drugs and factors of oxidative stress as well as some of the characteristics were associated with the cell lines. The FLIM-FRET of tumor xenografts showed the distribution of short-lived and long-lived components which also changed after administering some antitumor drugs. Changing of life-time distribution was not dramatic as on the cell level and depended on tissue properties as well.

Conclusions

FLIM-FRET based on genetically encoded biosensor is a highly informative tool for monitoring proteolytic activation in cells and in subcutaneous tumor xenografts as well. Changes in the distribution of life-time of TR23K biosensor depend mostly on the amount of cleaved DEVD caused by antitumor drugs and factors of oxidative stress.

Acknowledgements

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Neurobiophotonics

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SELECTIVITY OF THE NEURAL NETWORK OF THE PRIMARY HIPPOCAMPAL CULTURE TO THE ELECTRICAL STIMULATION

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Abstract. The coding of sensory information is a brain feature expressed as a certain structure of the neural network response. Decoding of the stimulus characteristics requires selective behavior of the neural network in response to the various input signals. In this study, we investigated the ability of the hippocampal neurons in a culture to generate the selective response to the various low-frequency stimuli spatially distributed in the network. We found that the fundamental evoked response features are the first spike time and the spike rate of the response, and they represent the unique parameters of the evoked response in the neural network.

Introduction

The cultured neuronal networks are perspective experimental models for the study of the cellular mechanisms of signal propagation and information processing at the network level. The networks generate the synchronized bursting events (of 0.5-2 s duration) with high frequency spiking elicited by a large number of cells involved in the network. The bursting activity in a culture was investigated in connection with learning in neural networks [1-2], signal processing at network level [3-4] and other problems.

The information processing in the neural networks requires efficient coding/decoding of the signals in spiking patterns. The ability of the neurons to decode information from spiking activity can be characterized by the so-called selectivity feature. The selectivity is the key property of the output information classification from the brain and is often investigated to decode stimulus specific information from the response of the neural network to the input electrical signals. In the present study we show that the selectivity feature in the cultured hippocampal neural network is formed by the categorization of stimulus location in the network on the basis of precise spike time and spike rate of the response to the electrical stimulus.

Materials and methods

We analyzed the activity of a neuronal culture on the multielectrode array (MED64, Alpha Med Sciences, Japan). Dissociated hippocampal cells were extracted from the brain of the mice embryos on the 18th prenatal day (E18) and cultured directly on 64-electrode arrays MED64 probe (neurons were plated with the density of 3000 cells/mm²) during 30 days at the temperature of 35.5°C in the atmosphere of 5% of CO₂ in Neurobasal medium with B27, L-glutamine and fetal calf serum. No antibiotics or antimycotics were used.

Electrical low-frequency stimulation (0.05-0.3 Hz) from the pairs of the close electrodes (stimulation sites) was applied using a stimulus generator. The stimulation consisted of the bipolar pulse train with a peak current of 50 μ A and a duration of each pulse of 500 μ s and was applied to two stimulation sites. These two stimulation sites were randomly chosen for each experiment according to its ability to induce the population bursts in a response to more than 80% of the stimuli from both sites. Each site was stimulated for 10 min.

Results

Using the dissociated hippocampal cultures grown on multielectrode arrays, we studied whether the low-frequency electrical stimulation applied to the different sites can evoke statistically significant spiking patterns in the neuronal networks. We analyzed spiking activity evoked by the consecutive stimulation of the two pairs of the electrodes. The network response typically consisted of the short interval (200-500 ms) burst-activity generated by the neurons. We analyzed the selectivity of the neurons at a single electrode. We defined the selectivity as the ability of the neurons of the network to generate different patterns of activity in response to different stimuli. Responses at each electrode can be characterized by two main parameters: time of appearance of the first spike after the stimulus artifact and the total spike rate of the response within the first 500 ms period of the post-stimulus interval. Analysis was performed only for the electrodes which responded to more than 80% of the stimuli from

both stimulation sites and were determined as active electrodes. We analyzed characteristics of the responses to stimuli as the first spike time after stimulus and the total spike rate for 500 ms after stimulus artifact for each electrode. If the values from the two sets of the total spike rate or the first spike time were statistically different, the neurons on the electrode were considered to be statistically selective to the stimulation site. The summarized results of the statistical selectivity of a single electrode are shown in Fig. 1.

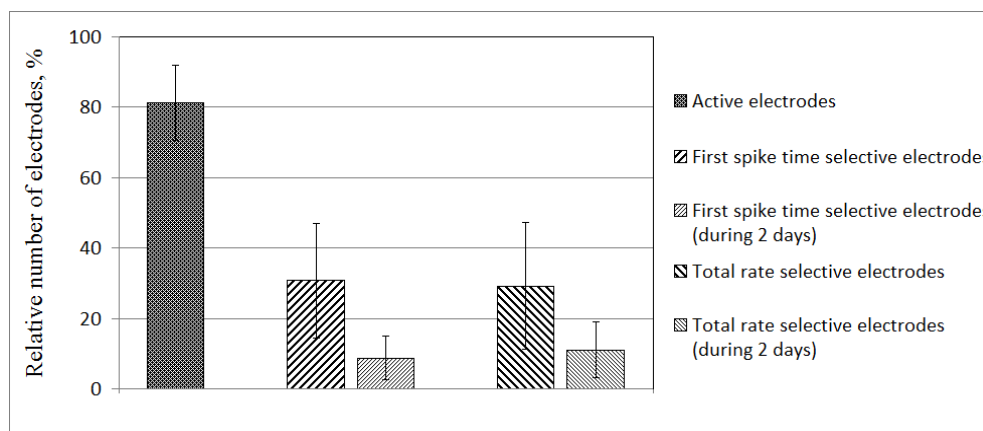


Fig. 1. Relative number of active electrodes, of the electrodes selective to the stimulus source using the total spike rate and the first spike time as response characteristics

The relative number of active electrodes was $81.25\% \pm 10.78\%$ on the average. The results show that the relative number of the selective electrodes using the first spikes times was $30.73\% \pm 16.31\%$, and the relative number of the selective electrodes using spike rates was $29.13\% \pm 17.99\%$. The selective electrodes make up 37% of the active electrodes. It was shown that approximately 10% of the electrodes maintained their selectivity for 2 days (the selective electrodes using the first spike times – $8.77\% \pm 6.30\%$ and the electrodes using the spike rates – $11.06\% \pm 8.03\%$).

Conclusion

It was shown that the small fraction of the neurons of the primary hippocampal culture demonstrate selectivity to different external electrical stimuli. This selectivity appears on particular electrodes (e.g. neuron group) that can reliably distinguish the type of the stimulus in its spatial location. Such high selectivity of the neurons to individual electrodes was verified using the first spike timing and the total spike rate. We demonstrated that on the average the number of electrodes with the statistically significant selectivity using the first spike timing characteristics is greater than the number of electrodes using total spike rate characteristics but not significantly.

Thus, our experiments on the electrical stimulation of the neuronal culture reveal the existence of the mechanisms of formation of different responses of neural networks to the electrical stimuli. We have shown that there are neurons which are capable to perform the classification of the input signal during stimulation. The evoked response dynamics indicates that the culture networks contain different signaling pathways activated selectively by the appropriate stimulus. The response selectivity with the robust statistical characteristics can be further used to design the closed-loop solutions of the culture network based control systems (e.g. neuroanimats) where the selective electrodes which generate distinguishable responses are associated with different sensory signals.

Acknowledgements

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SPONTANEOUS REVERBERATIONS IN DEVELOPING NEURONAL CULTURES

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Abstract. Spontaneous synchronized bursting in a developing neuronal network is studied by a multi-electrode array system. The synchronized burst is characterized by the histogram of spiking time averaged over 60 electrodes. We revealed that reverberations can be found from these histograms which are studied as a function of day in vitro and pharmacology. Our results show that the observed changes in reverberations of the network originate from the changes in network connectivity in the culture.

In many experiments with neuronal cultures, the dynamics emerging from a network of excitable elements is often a synchronized activity; such as the synchronized bursting found in neuronal cultures. Such spontaneous synchronized activity is an important phenomenon in developing neuronal cultures. Recent studies revealed that the firing patterns play an important role in the network developing and memory. It is believed that the dynamics of firings is related to network structures. However, it is still unclear how the firing patterns are related to network structure. In this study, we measure the firing patterns of cultures in a MEA system at different DIV to study the relation between dynamics and structures with the help of pharmacology.

Primary cortical cells (E17) were dissociated and plated onto polyethyleneimine coated micro electrode arrays (8×8 with 200 μm spacing; Ayanda Biosystem) with cell density of 3000 cells/ mm^2 . Cultures developed in DMEM with 5 % horse serum and 5 % Fetal bovine serum and were incubated in 5 % CO_2 and 37 $^\circ\text{C}$. The activities of neurons were recorded for 10 minute by MEA 1060-INC-BC (Multi Channel System) with a sampling rate of 20 Hz at 37 $^\circ\text{C}$ and 5 % CO_2 . Spikes were detected when the signal was greater than seven times of standard deviation of noise.

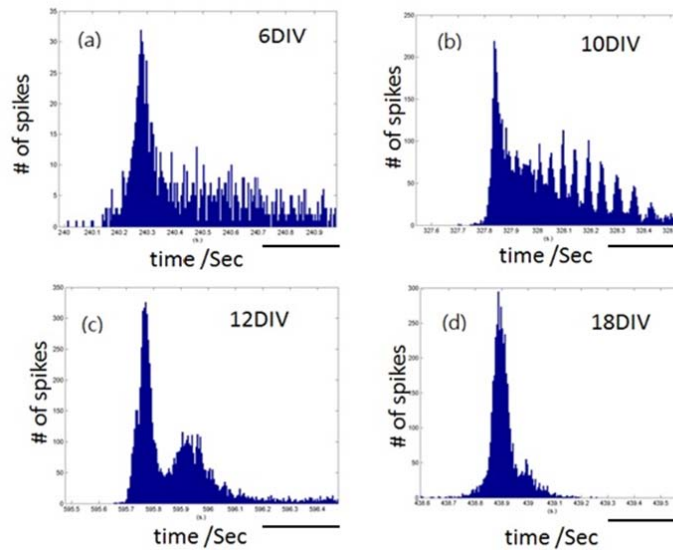


Fig. 1. Histograms of spontaneous activities within synchronized burst at a) 6 DIV, b) 10 DIV, c) 12 DIV and d) 18 DIV. Scale bar is 200 ms

A synchronized burst is characterized by the histogram of spiking time averaged over 60 electrodes with 5 ms time bin. These histograms were studied as a function of DIV (Fig. 1) and pharmacology (Fig. 2). Histograms were measured in different buffer solutions: magnesium free buffer, bicuculline methiodide (BMI) containing buffer and Glutamate containing buffer.

Synchronized bursts with duration of the time scale of seconds can be observed one week after plating. As the cultures mature, the time interval between burst and burst duration becomes shorter. At early DIV, the structure of the histograms shows that the neurons are firing more or less periodically within a burst with the time interval of 100 ms; similar to reverberation [2]. Figure 1 shows the changes

of the histograms as a function of DIV. It can be seen that the periodic structures of the histogram disappear around 12 DIV which can be culture dependent.

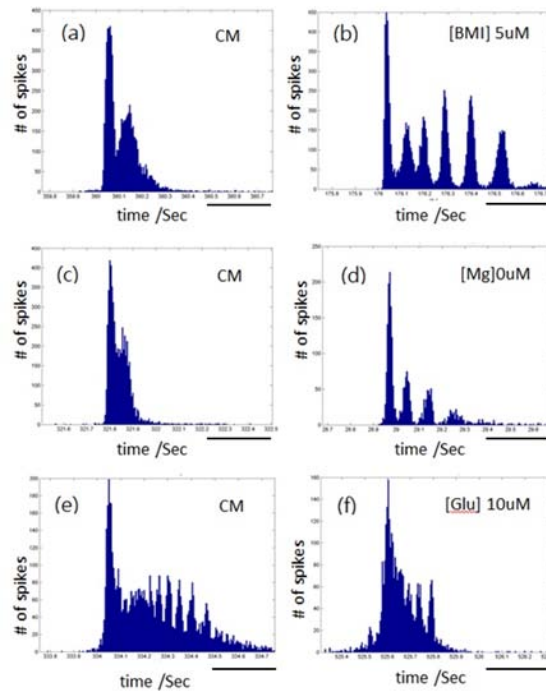


Fig. 2. Results of pharmacology studies of spontaneous activities within a synchronized burst. a) and b) are the histograms with 0 and 5 μ M BMI respectively at 24 DIV. c) and d) are the histograms in culture medium and BSS with no Mg respectively at 27 DIV. e) and f) are the histograms with 0 and 10 μ M glutamate respectively at 10 DIV. Scale bar is 200 ms

Bicuculline is a blocker of inhibitory transmission (GABA A receptor antagonist). Figure 2 (a) and (b) are the histograms before and after additional BMI. It shows that blocking inhibitory transmission enhanced the reverberation and extended the burst duration time. The concentration of magnesium is related to the synaptic connectivity of the cultures[3]; a decrease of [Mg] can be considered as an increase of network connection. Figures 2 (c) and (d) shows that the reverberation can be induced by the removal of magnesium from the buffer. The addition of glutamate is used to increase the random firings of neurons to simulate an increase of environment noise. Figures 2 (e) and (f) show the reverberation suppressed by the addition of Glu.

Summary

Our experiments show that 1) network activities change during network development. 2) Reverberation can only be observed at 13 ± 3 DIV. 3) Results of BMI and Mg suggest that the reverberation is the result of a better connected network. 4) Results of glutamate experiments show that an increase of system noise effectively reduces the connectivity of the system. 5) The disappearance of reverberation after 17 DIV suggests that either i) there is substantial increase in noise or ii) there is a decrease in connectivity in the network.

Acknowledgements

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LINKING BIOLOGICAL AND ARTIFICIAL SYSTEMS: TOWARDS THE FUTURE INTEGRATION OF BRAIN AND MACHINES

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Abstract. Behaviors, from simple to most complex, require a two-way interaction with the environment and the contribution of different brain areas depending on the orchestrated activation of neuronal assemblies. Here we present a new hybrid neuro-robotic architecture based on a neural controller bi-directionally connected to a virtual robot implementing a Braitenberg vehicle. The robot is characterized by proximity sensors and wheels, allowing it to navigate within an arena with obstacles. As neural controller, we used neocortical cultures kept alive over Micro Electrode Arrays. The proposed neuro-robotic framework provides a suitable experimental environment for studying the interaction between brains and machines.

Introduction

In recent years, there has been a growing recognition of the crucial role played by an animal's body and the surrounding environment in understanding the neural basis of behavior [1]. According to this view, behavior arises through the interaction of neural activity, body and environment. The environment itself can be manipulated, and the corresponding changes in dynamic behavior can provide useful information for understanding the neural systems themselves. For the above reasons, researchers have begun to explore the possibility to create new in vitro systems at the interface between neuroscience and robotics. At the same time, these systems can provide excellent test beds for electrical training and modulation of neuronal tissue, thus forming the basis of future adaptive, closed-loop Brain Machine Interfaces [2].

The first-ever in vitro closed-loop system was developed at Northwestern University [3]: it consisted of a lamprey brain-stem bi-directionally connected to a small robot. The main goal of our research is to reproduce that experimental 'neuro-robotic' framework by using a different brain model (i.e. neocortical cultures dissociated from embryonic rats and kept alive over Micro Electrode Arrays), which can be easily 'engineered' and manipulated, either chemically or electrically. To this end, we developed a software architecture which guarantees a bi-directional exchange of information between the natural and the artificial part by means of simple linear coding/decoding schemes.

Materials and Methods

The neuro-robotic architecture includes several different elements:

1. A network module, constituted by a neuronal culture over a Micro Electrodes Array - MEA (i.e. the 'brain' of the neuro-robotic loop).
2. A computer, equipped with a data acquisition board, which hosts the developed software able to manage all the devices included in the architecture.
3. A stimulation unit, which is able to handle two different stimulation patterns. The stimulation signals are programmed via software and they are defined by their frequency, amplitude and stimulation site.
4. A robotic module, characterized by a small robot (either physical or virtual) with sensors and wheels able to move inside a circular arena with obstacles.

These different modules are synchronized and managed by a custom developed software named HyBrain which runs in the Windows environment [4]. Through this software, it is possible to control the parameters of the neuro-robotic experiments, namely the coding, decoding and learning schemes and all the required data processing.

Neuronal cultures

Dissociated networks of neurons represent an intermediate level of organization of the nervous system and they constitute a valuable experimental model for studying the collective electrophysiological/functional properties of the brain. MEAs, able to detect the activity of the networks at different recording sites, allow measuring the cultures from many sites (e.g. 60) and for long periods (weeks, months). During the in vitro development [5, 6], synapses start to be established and neurons begin to communicate with each other, building a 2D network. Networks freely develop and basic electrophysiology

siological, biochemical and pharmacological properties are exhibited similar to those of *in vivo* neurons.

In this study, we used two different kinds of experimental preparation: ‘homogeneous’ (i.e. random) and “modular” networks. The “modular” networks have been obtained by the use of a dual-compartment chamber with two interconnecting microchannels in PDMS (polydimethylsiloxane), a biocompatible, inert and non-toxic polymer. The modular structures have been realized by replica molding using specific master with a previously developed technique. The obtained structures have been then placed on MEA substrates, in order to confine the growth of the neuronal cells that were plated on it afterwards.

Results

The first step of our experiments consists of the characterization of the network under study, both in spontaneous and evoked activity. To this end, we demonstrated that homogeneous and modular networks show a different dynamics both during development and under stimulation. We analyzed the bursting activity, which is a peculiar feature of these networks, by detecting the “major burst leaders” (MBL) and highlighting the differences between the two kinds of preparations. Then, by selecting specific “input” (i.e. sensory) and “output” (i.e. motor) areas in every network, we were able to successfully interface it with an artificial agent in a bi-directional way. Once controlled by the neuronal network, the robot was able to navigate into an arena with obstacles. While collisions were fairly frequent even in the case of a neuron-controlled robot, the global behavior of the robot was still much closer to the desired one rather than in an open-loop configuration, or in the absence of a biological substrate. Finally, we found that the robot performed better if a reinforcement learning paradigm (i.e. a tetanic stimulation delivered to the network following each collision) was activated.

Conclusions

Our results prove that a culture of dissociated neurons has very rich dynamics and exhibits basic mechanisms of learning similar to those observed in *in vivo* systems. Thanks to these features, it can be successfully interfaced in a bi-directional way with a robot with sensors and actuators. The presence of a robot bi-directionally connected to a simple neuro-controller based on living neurons allows performing experiments in the context of the embodied electrophysiology paradigm, providing time-varying stimuli, testing learning schemes and managing sensory feedbacks in a more realistic environment. Our neuro-robotic framework can be exploited in order to study the mechanisms of neural coding and the computational properties of neuronal assemblies with the final goal to facilitate progress in understanding neural pathologies, designing neural prosthetics, and creating fundamentally different types of artificial intelligence

Acknowledgements

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COMPARING PROTEIN REPORTERS OF MEMBRANE POTENTIAL AND CALCIUM AS INDICATORS OF ODORANT RESPONSES IN THE *IN VIVO* MOUSE OLFACTORY BULB

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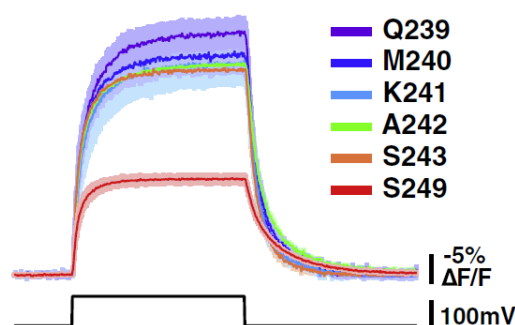
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Abstract. In principle, protein reporters of activity allow for recording of genetically distinct population of neurons. However, the usefulness of protein reporters of membrane potential has been limited by poor *in vivo* expression, small signal sizes, and/or slow kinetics. The novel fluorescent protein (FP) voltage sensor ArcLight (based on the *Ciona* voltage sensitive phosphatase voltage sensing domain (Dimitrov, et al, 2007)) exhibits a change in fluorescence to a 100 mV depolarization five times larger than previously reported probes (Jin, et al, 2012). Previous recordings using ArcLight were limited to HEK 293 cells and cultured neurons. We examined ArcLight responses in the olfactory bulb of an *in vivo* mouse preparation and compared them to the responses of the genetically encoded calcium indicator GCaMP3.

Figure 1 illustrates the response of ArcLight to a 100 mV depolarizing voltage step in HEK293 cells. The depolarization elicits a 40% change in fluorescence. The onset and offset are reasonably well fit with a single exponential of 20-30 msec.

Fig. 1. Fluorescence response of several ArcLight FP voltage sensors with varying linker lengths. The depolarization duration was 300 msec. A single trial was recorded from each cell (n= 5-8) and the optical traces of these trials were averaged. From Jin et al (2012)



Together with the UPenn virus facility we made a AAV1 ArcLight vector (Fig. 1, A242) and injected this virus into one mouse hemi-olfactory bulb. We injected an AAV1 GCaMP3 virus (obtained from the UPenn facility) into the other hemi-bulb. Figure 2 shows the response to odorant of ArcLight (red) and GCaMP3 (green).

Odorant response (ethyl tiglate) of ArcLight and GCaMP3.

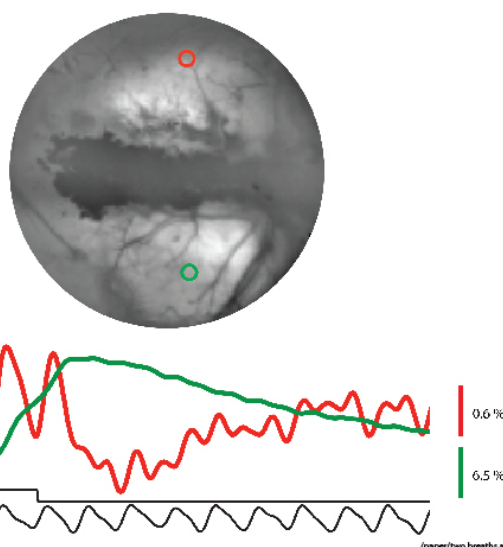


Fig. 2. Response to a 0.6 second odorant exposure from two mouse hemi-olfactory bulbs one expressing ArcLight (red) and the other expressing GCaMP3 (green). The recording is the average of four trials. (Douglas Storace, Lawrence Cohen, and Uhna Sung' preliminary results)

Although odorant-specific patterns of activation were obtained from both ArcLight and GCaMP3, only ArcLight had sufficiently fast temporal kinetics to clearly detect the population activity elicited by individual breaths (Fig. 2c, red versus green trace). ArcLight is a reliable detector of odor-evoked population signals in the mouse olfactory bulb.

Acknowledgements

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MEMORY IN CULTURED CORTICAL NETWORKS: EXPERIMENT AND MODELING

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Abstract. The mechanism behind memory is one of the mysteries in neuroscience. Here we unravel part of the mechanism by showing that cultured neuronal networks develop an activity connectivity balance. External inputs disturb this balance and induce connectivity changes. The new connectivity is no longer disrupted by reapplication of the input, indicating that a network memorizes the input. A different input again induces connectivity changes, but returning to the first input no longer affects connectivity, showing that memory traces are stored in parallel. Computer modeling supports these findings, and shows that spike timing dependent plasticity enables neuronal networks to store memory traces of different inputs in parallel.

Introduction

Dissociated cortical neurons cultured on multi electrode arrays have received increasing attention to study network aspects of neuronal tissue, including memory. In the first week of culturing networks are formed. After ~ 1 week networks become spontaneously active and reach a mature state after ~ 3 weeks, with relatively stable activity patterns. Activity patterns are determined by a certain connectivity, and conversely, certain patterns also affect connectivity through plasticity mechanisms like e.g. spike timing dependent plasticity (STDP). Beyond three weeks, networks appear to develop an activity-connectivity balance, wherein occurring activity patterns support current connectivity. Responses to electrical stimulation usually differ from spontaneously occurring patterns and therefore disturb the activity connectivity balance, yielding a change in connectivity. We investigated the effect of different inputs on connectivity.

Methods

Cell culturing

We obtained cortical cells from newborn Wistar rats. About 400,000 dissociated neurons (400 μ l suspension) were plated on a MEA, resulting in a cell density of approximately 2500 cells per mm^2 .

Neurons were cultured in a circular chamber glued on top of a multi electrode array (MEA) with 60 electrodes (Multi Channel Systems, Germany). MEAs were stored in an incubator, under standard conditions. During recording we maintained the CO_2 level of the environment around 5%. For details about the recording setup see [1]. All recordings were started after an accommodation period of at least 10 minutes. After the measurements the cultures were returned to the incubator.

We used 9 different cultures for 19 experiments, which were performed 22 ± 6 days after plating of the dissociated cells.

Connectivity analysis

We used periods of spontaneous activity to analyze network connectivity. Long term recordings were divided into data blocks of 2^{13} spiking events. In each data block we used conditional firing probabilities to determine functional connectivity [2]. For all possible pairs of electrodes (60×59) we calculated conditional firing probabilities (CFP's) as the probability to record an action potential at electrode j at $t=\tau$, given that one was recorded at electrode i at $t=0$. If a CFP curve was not flat, the two neurons were functionally connected. This functional connection may be described by two parameters: strength and latency [2]. These parameters may be used to follow the development of a functional connection in time [3].

In each data block, the strengths of all connections were combined into a connectivity matrix S , with $S(i,j)$ the strength of the functional connection from i to j . The magnitude of changes between subsequent data blocks was assessed by the Euclidean distance between connectivity matrices.

Modeling

We constructed a computational model that consisted of 100 neurons, following the approach by Izhikevich [4]. The model contained a random mixture of all cell types that exist in the cortex, just like the experimental cultures. 80% of the neurons was excitatory, 20% inhibitory. These neurons were

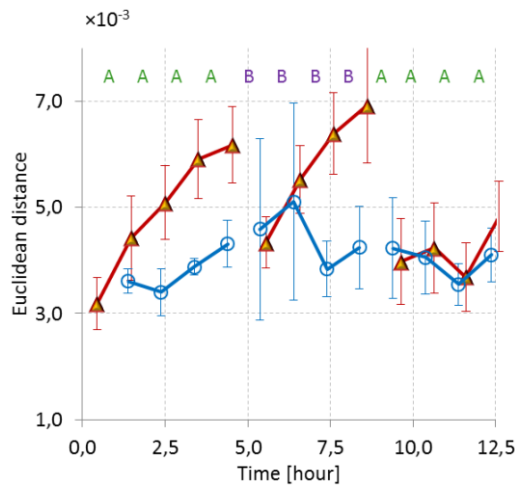


Fig. 1. Euclidean distance between subsequent connectivity matrices without stimulation ($\circ$, $n=4$ experiments), or across periods of tetanic stimulation at electrode A or B, as indicated (Δ , $n=7$ experiments). For comparison, all distances are relative to the connectivity immediately before stimulation at that electrode (A or B)

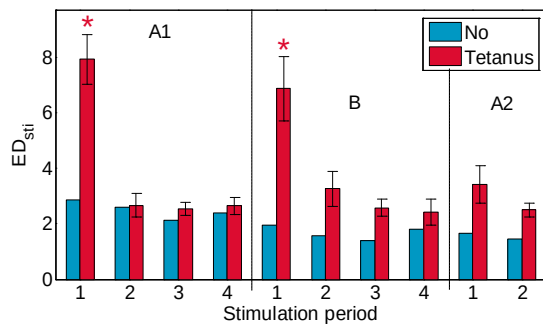


Fig. 2. Euclidean distances across subsequent stimulation periods at electrode A or B ($n=4$ simulations), or periods of no stimulation ($n=1$). Significant differences are indicated by *

Discussion

We concluded that cortical networks memorize inputs. External input drives the network out of the existing activity $\leftrightarrow$ connectivity balance. A new balance develops, probably including the response pattern to that stimulus. Consequently, following inputs on the same electrode had no effect on connectivity. A similar pattern occurred upon stimulation at another electrode. Returning to a previously applied stimulus did not change network connectivity, indicating that memory traces exist in parallel. Computational modeling suggests STDP as a crucial factor for this type of memory.

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coupled by synapses that displayed short term depression [5] and spike timing dependent plasticity [6]. On average, all neurons had 50 connections. Spontaneous activity was initiated by white synaptic noise, stimulation was simulated by imposed simultaneous firing of a randomly selected set of 7 neurons.

Results

In 19 *in vitro* experiments we showed that: 1) without external input, functional connectivity was stable (strengths varied less than 25% of their mean value) at time scales of multiple hours, 2) functional connectivity changed significantly after 10 minutes of electrical (tetanic) stimulation, 3) repeated application of the same stimulus induced much smaller or even negligible connectivity changes, and 4) stimulation at another electrode yielded large changes upon first stimulation and also smaller or no changes after succeeding stimuli. 5) Returning to the first stimulus did not induce connectivity changes larger than spontaneous fluctuations, as illustrated by Figure 1. Differences across first stimulation periods were significantly larger than those across subsequent stimulation periods, or periods of no stimulation.

A model of 100 neurons coupled by synapses with short term depression and spike timing dependent plasticity, robustly reproduced the findings that networks develop an activity-connectivity balance and that a first external stimulus (10 min, tetanic) induced large connectivity changes, but subsequent stimuli did not. This also applied for a second (different) stimulus, provided that synaptic strengths did not reach extreme values (maximum strength or zero). Return to the first stimulus did not induce changes larger than spontaneous fluctuations, see Figure 2.

ULTRASTRUCTURAL CORRELATES OF FUNCTIONAL NETWORK ACTIVITY OF HIPPOCAMPAL NEURONS DEVELOPING IN VITRO

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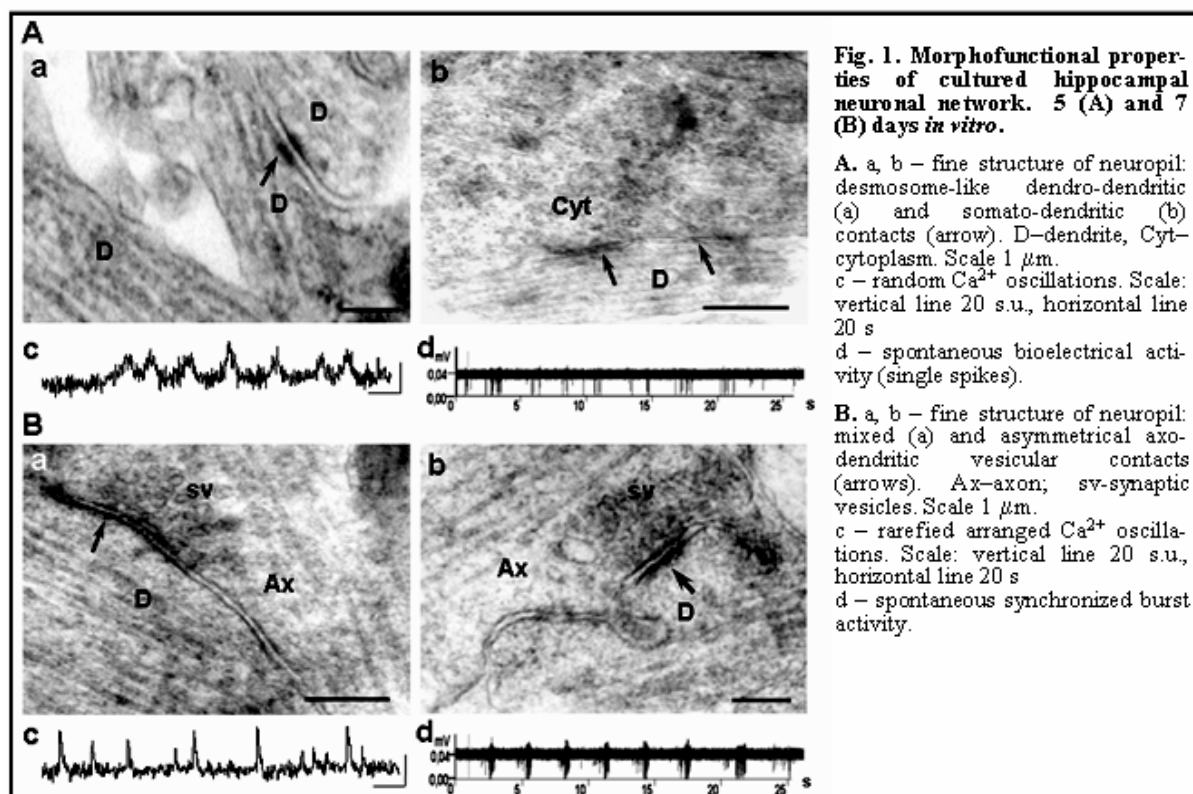
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Abstract. An ultrastructural study of synaptogenesis during the morphofunctional development of cultured mice embryonal hippocampal cells for 3-4 weeks *in vitro* showed gradual maturation of synaptic contacts accompanied by complication of functional calcium imaging and spontaneous network spike activity (appearance of calcium “superoscillations” and spike “superbursts”, respectively). The ultrastructure of interneuronal connections, as well as the patterns of neuronal network functional activity were stabilized during a period of one month. The obtained results testified direct correlation between dynamics of maturation of excitatory synapses and complication of functional properties of cultured hippocampal neuronal network.

Though the main features of development of cultured hippocampal cells have been thoroughly studied [1], the correlations of ultrastructural changes of the developing interneuronal contacts with the dynamics of functional network activity *in vitro* is still unclear. The aim of the investigation was to study the development of synapses in primary hippocampal cell culture and functional patterns of cultured hippocampal neuronal network.

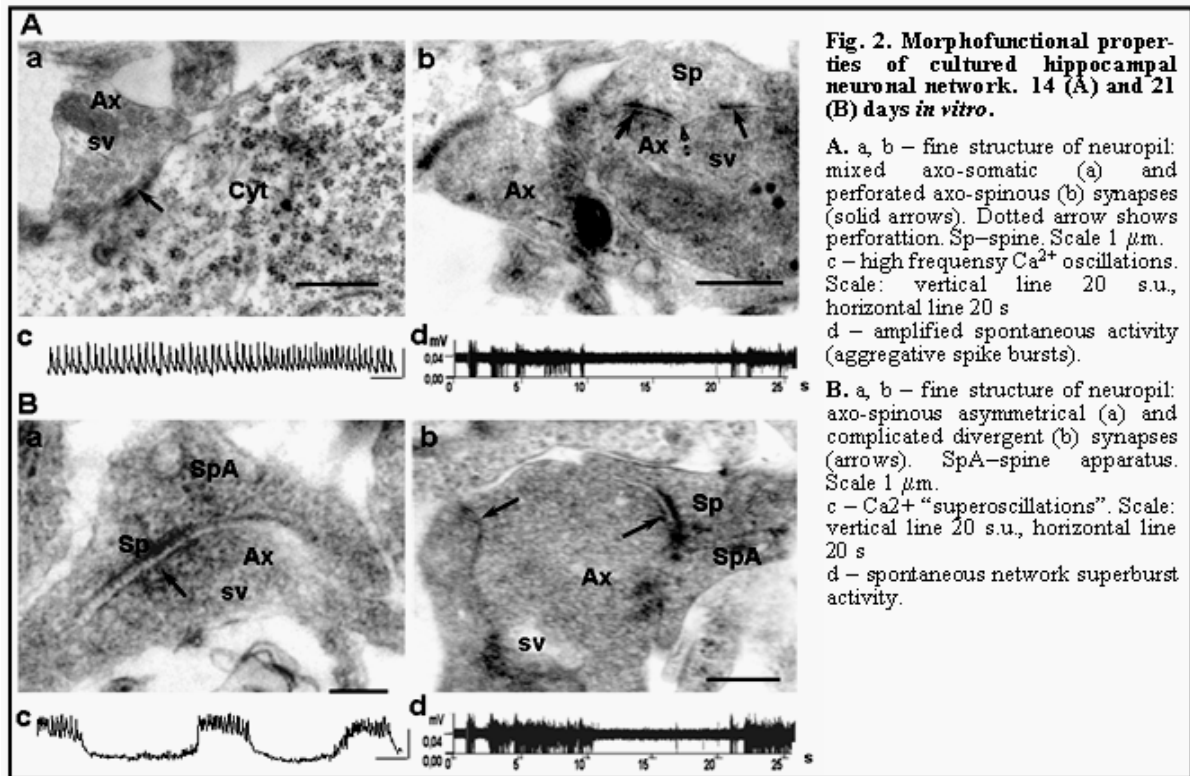
We performed experiments on long-term (up to 1 month) cultures of hippocampal cells from 18-day mice embryos [2]. For ultrastructural investigations, stained ultrathin sections of cultures were investigated in electron microscope. The neuronal Ca^{2+} imaging was monitored by measuring the changes of intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) using specific fluorescent calcium probe Oregon Green BAPTA-1 AM, and analyzed with MATLAB-based software [3]. Spontaneous network activity was recorded using multielectrode array system MED 64 (Alpha MED Science, Japan).

Ultrastructural analysis of 5 days *in vitro* (DIV) cultures revealed in neuropil vacant postsynaptic densities and a large amount of immature desmosomal non-vesicular contacts which are atypical for mature brain junctions (Fig. 1A, a, b) but able nevertheless to transmit some electrical signals [1]. In single neurons, random spontaneous Ca^{2+} -oscillations with low frequency and large-scale width were observed (Fig. 1A, c). In spontaneous activity the single low-frequency spikes predominated (Fig. 1A, d).



At 7 DIV, desmosomes formed mixed contacts (Fig. 1B, a) representing an intermediate stage of development of a chemical synapses. The first mature synapses were asymmetric axodendritic (Fig. 1B, a, b), and their presynaptic terminals contained numerous synaptic vesicles. The frequency of Ca^{2+} oscillations increased, their width decreased (Fig. 1B, c), and the number of neurons generating these oscillations increased. In patterns of network activity short-time synchronized spike bursts were detected (Fig. 1B, d).

In two weeks *in vitro*, typical symmetrical (inhibitory) axosomatic and numerous asymmetrical (excitatory) mature synapses appeared (Fig. 2A, a). Among them, axospinous contacts, including perforated ones, which might increase neurotransmission efficiency [4], were revealed (Fig. 2A, b). Ca^{2+} oscillation width decreased, whereas their frequency continued to increase (Fig. 2A, c). In this period, spontaneous network activity became forced, and aggregative spike bursts were recorded (Fig. 2A, d).



After 21 DIV, some complicated axospinous, perforated, divergent and convergent contacts, as well as the elements of spine apparatus in some spines were found (Fig. 2B, a, b). Among single Ca^{2+} oscillations, the “superoscillations” consisting of a large number of single oscillations were monitored (Fig. 2B, c). Network activity continued to complicate and at 30 DIV became to be stable, with predomination of “superbursts” containing numerous integrated spikes (Fig. 2B, d).

Thus, dissociated hippocampal culture can be considered as an adequate biological model of brain neuronal network development. Maturation of cultured hippocampal neuronal network occurs in the period from 21 to 30 DIV, when the main population of excitatory synapses consists of mature axospinous asymmetric contacts, and the cultures are characterized functionally by stable synchronous burst activity.

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CONNECTIVITY MOTIFS IN NETWORKS OF MODEL NEURONS WITH PLASTIC SYNAPSES

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Recent evidence in rodent cerebral cortex and olfactory bulb [1, 2] suggests that short-term dynamics of excitatory synaptic transmission is correlated to stereotypical connectivity motifs. It was observed that neurons with short-term facilitating synapses form predominantly reciprocal pairwise connections, while neurons with short-term depressing synapses form unidirectional pairwise connections. The cause of these structural differences in synaptic microcircuits is unknown.

We propose that these connectivity motifs emerge from the interactions between short-term synaptic dynamics (SD) and long-term spike-timing dependent plasticity (STDP). While the impact of STDP on SD was demonstrated *in vitro*, the mutual interaction between STDP and SD in large networks is still the subject of intense research. We formulate a computational model by combining SD [3] and STDP [4], which captures faithfully short- and long-term dependence on both spike times and frequency. As a proof of concept, we simulate recurrent networks of spiking neurons with random initial connection efficacies and where synapses are either all short-term facilitating or all depressing. For identical background inputs, and as a direct consequence of internally generated activity, we find that networks with depressing synapses evolve unidirectional connectivity motifs (Figure 1), while networks with facilitating synapses evolve reciprocal connectivity motifs (Figure 2). This holds for heterogeneous networks including both facilitating and depressing synapses.

Our study highlights the conditions under which SD-STDP might explain the correlation between facilitation and reciprocal connectivity motifs, as well as between depression and unidirectional motifs. An earlier preprint of this work was submitted to Cornell University Library archive [5].

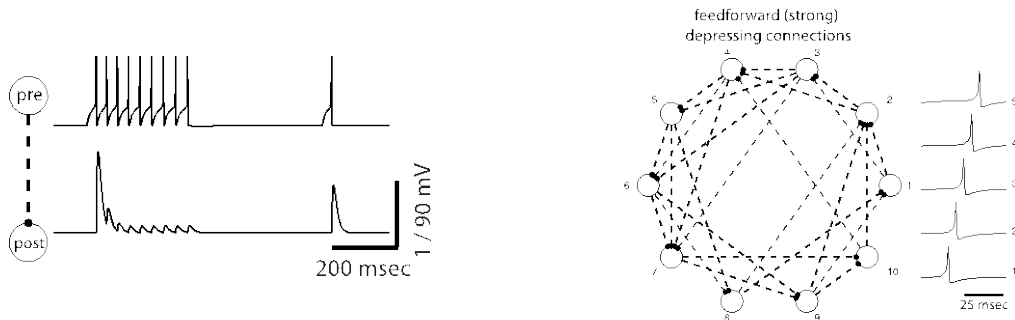


Fig. 1. A “toy” network of ten exponential Integrate-and-Fire excitatory model neuron, connected by (left) short-term depressing synapses, is computer simulated. The long-term network effective synaptic connectivity converges (right) to a largely feed-forward topology

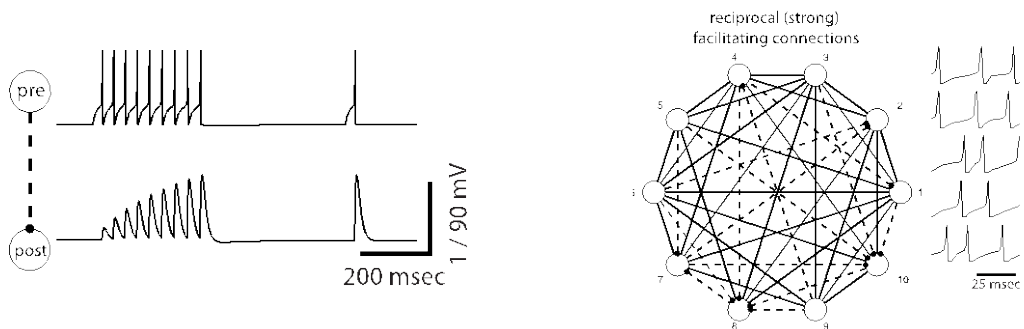


Fig. 2. The same model network of Figure 1 is simulated under exactly the same external input, with model neurons are connected by (left) short-term facilitating synapses. The long-term network effective synaptic connectivity converges to (right) a largely recurrent topology

Acknowledgements

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ANALYSIS AND CONTROL OF CULTURED NEURONAL NETWORKS USING MULTI-ELECTRODE ARRAYS: FROM GENE EXPRESSION TO NETWORK DYNAMICS

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Abstract. Using multi-electrode arrays (MEAs), we investigated the network dynamics of cultured neuronal cells. We measured spontaneous electrical activity of the networks at least 1 month. Over this culture period, the network showed an initial increase and subsequent saturation of the activity. As part of the study, the network components, such as the soma, glutamatergic synapses, and GABAergic synapses, were labeled immunocytochemically and analyzed quantitatively. In addition, the expression of neuron-specific genes was investigated. Here, we show our recent results using cultured neuronal networks and MEAs.

Previous studies using animal models and acute slices of brain tissue have revealed many mechanisms associated with nervous system development and function. *In vitro* culture systems have also contributed to the elucidation of molecular and cellular mechanisms. The biggest advantage of using a neuronal culture is that it simplifies the system. In addition, cultured neurons enable the long-term, continuous monitoring of the development and maturation of neuronal networks. Taking advantage of this system, we established long-term (1-2 months) cultures of neuronal cells derived from rat cortical cortex at embryonic day 17 and analyzed the cultured neuronal networks. Multi-electrode arrays (MEAs) are very useful tools for recording the electrical activity of cultured neuronal cells. MEAs also facilitate long-term observation of network activity, since electrical signals can be observed without damaging the cells. Using MEAs, we analyzed the spatiotemporal dynamics of cultured cortical networks. Here, we present our group's recent results.

Long-term measurement and analysis of electrical activity using MEAs

Spontaneous electrical activity was recorded for at least 1 month using MEAs. The spontaneous spikes became synchronized bursts as the neuronal networks developed. To quantify the network activity, we calculated the network firing and synchronized burst rates. The network activity showed an initial increase and subsequent saturation of both rates during the 1-month culture period [1]. We also analyzed the electrical activity of cultured neuronal networks to clarify the details of the spatiotemporal dynamics [2].

Immunofluorescence imaging and quantitative analysis

To analyze spatial factors that affected the neuronal dynamics over the month, we performed immunofluorescence staining of neuron-specific proteins [1]. We labeled glutamatergic and GABAergic synapses separately using antibodies against vesicular glutamate transporter 1 (VGluT1) and vesicular transporter of γ -aminobutyric acid (VGAT), respectively. The densities and distributions of both types of synaptic terminal were measured simultaneously. Observations and subsequent measurements of immunofluorescence demonstrated that the densities of both types of antibody-labeled terminal increased gradually from 7 to 21-28 days *in vitro* (DIV). The densities had not increased further at 35 DIV and tended to become saturated. Triple staining with VGluT1, VGAT, and microtubule-associated protein 2 (MAP2) enabled analysis of the distribution of both types of synapse, and revealed that the densities of both types of synaptic terminal on somata were not significantly different, but that glutamatergic synapses predominated on the dendrites during long-term culture. In addition, the number of culture days to saturation from the initial increase corresponded to the electrical activity.

By contrast, the density of neurons labeled with MAP2 antibody decreased gradually over the culture period. Then, the density of surviving neurons remained constant from 35 to 60 DIV, and the neurites labeled with neurofilament 200kD (NF200) covered the surface of the culture up to 60 DIV. These results indicate that cultured neuronal networks survived at least 2-month culture periods.

Gene expression analysis

The molecule mechanisms involved in the generation and maintenance of synchronized bursts in cultured neuronal networks remain unclear. Although minute-to-hour time-scale changes in gene and protein expression during pharmacologically induced activity changes in synchronized bursts have been investigated, the molecules involved in network construction and the subsequent generation of synchronized bursts during long-term development (day-to-month time-scale changes) remain unclear. This phenomenon can be elucidated by combining MEA-based non-invasive recordings of electrical activity with molecular-level studies during long-term neuronal culture. We focused on neuron-specific *Arc* gene expression, an immediate-early genes, and investigated the temporal relationship between *Arc* gene expression and the generation of synchronized bursts over 1-month culture. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that the transcription factor gene *Creb1* was consistently expressed during the culture period. In contrast, *Arc* gene expression was not observed at 1-7 DIV, but the gene was expressed from 14 to 35 DIV. Furthermore, the first expression of the *Arc* gene occurred at the same number of culture DIV as the generation of synchronized bursts under this culture condition. Based on these results, we have begun to investigate whether the generation of synchronized bursts correlates with the expression of *Arc* genes. In addition, other molecules should be investigated exhaustively to elucidate the molecular mechanisms underlying the generation of synchronized bursts.

Control of cultured neuronal networks

Generally, to obtain electrical signals from the electrode, neuronal cells are dispersed on MEAs at high densities. This requires analysis of many signals from a complex neuronal network, although the majority of cells are not involved and the signals of most other cells cannot not be detected in high-density culture. Patterning neuronal cell on MEAs is one approach to overcome this problem. We patterned Poly-D-lysine (PDL) with a photolithographic method using vacuum ultraviolet light (VUV) [3]. Primary neuronal cells were patterned without manipulating the cells on MEAs. The patterned neuronal cells showed synchronized bursts, suggesting that they had matured and were sufficiently developed under this condition [4]. This patterning method is useful for analysis of neuronal dynamics.

As a novel method, we used the gas anesthesia to control the cultured neuronal networks. Xenon (Xe) is an inert gas that produces general anesthesia without causing undesirable side effects. Under an applied Xe pressure of about 0.3 MPa and at physiological temperatures, the synchronized bursts ceased quickly, whereas single spikes continued. The Xe-induced inhibition-recovery of neuronal network firing was reversible: after purging Xe from the system, the synchronized bursts resumed gradually. Therefore, Xe did not inhibit single-neuron firing, yet it reversibly inhibited the synaptic transmission [5].

Acknowledgements

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NEUROPROTECTIVE PROPERTIES OF CANNABINOID N-ARACHIDONOYL DOPAMINE IN HIPPOCAMPAL NEURAL NETWORK CULTURED ON MULTIELECTRODE ARRAYS

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Abstract. We studied the neuromodulatory effect of synthetic endocannabinoid analog, N-arachidonoyl dopamine (N-ADA), on spontaneous bioelectric activity of mice hippocampal neural network cultured on multielectrode arrays and exposed to hypoxic lesion. Hypoxia and subsequent reoxygenation induced irreversible suppression of spontaneous burst activity followed by nerve cell death. The application of N-ADA (2.5-10 mcM) during and after hypoxic period preserved bioelectric activity and cell viability. The investigation of N-ADA protective effect mechanisms revealed not only preferential protective role of cannabinoid receptor type 1 (CB1-R), but also involvement in this effect of transient receptor vanilloid type 1 (TRPV1).

The modulation of neuronal spontaneous network activity is one of important problems in modern neurobiology. The most extended neurotransmitter which realizes the information exchange in brain neuronal networks is glutamate. At the same time, in some pathological conditions (e.g., in hypoxia/ischemia), hyperstimulation of postsynaptic glutamate receptors on account of strengthened glutamate release from presynaptic terminals (so-called glutamate excitotoxicity) results in abundant intracellular calcium accumulation inducing pathogenic cascade of lipo- and proteolytic reactions and causing neuronal death [1]. Therefore, neuromodulatory regulation of glutamatergic network neurotransmission may contribute to removal of negative consequences of its disturbance. Effective neuromodulators of excitatory synaptic activity are endogenous cannabinoids (EC), since their retrograde interaction with presynaptic cannabinoid receptors may inhibit the excessive release of neurotransmitters from axon terminals reducing intracellular calcium overload. One of the synthetic EC analogs, N-arachidonoyl dopamine (N-ADA), was recently identified as an neuroprotective endogenous capsaicin-like compound [2], which is present in mammalian brain and has a heterogeneous pharmacology [3], since it displays nanomolar potency not only for type 1 and, probably, type 2 cannabinoid receptors (CB1-R and CB2-R), but also for transient receptor vanilloid type 1 (TRPV1) channel as a non-selective cation channel.

We studied the effects of N-ADA on viability and spontaneous activity of mice hippocampal neural network cultured for 21 days *in vitro* (DIV) on multielectrode arrays [4] and exposed to hypoxia (incubation of cultures for 10 min in deoxygenated medium).

It was shown that the quantity of living cells considerably decreased in post-hypoxic period (Table 1). Immediately after hypoxia a burst quantity was increased (Fig. 1A), whereas a spike quantity in burst did not change (Fig. 1B). But the burst quantity gradually decreased after 2 hrs and 1 DIV of hypoxia and dramatic collapse of network activity at 7 DIV as well as decrease of spike quantity in burst were observed (Fig. 1A, B). Thus, these results suggested that short hypoxia and subsequent reoxygenation induce irreversible suppression of spontaneous burst activity followed by nerve cell death.

The application of N-ADA during and after the hypoxia prevented cell death and the disturbance of bioelectric

Experimental groups	Quantity of living cells (%)	
	1 DIV	7 DIV
Intact (normoxia)	89,37±1,33	89,02±2,11
Hypoxia	73,19±3,95 *	30,84±4,77 *,**
Hypoxia+N-ADA	75,13±3,72 *	75,24±10,37 *
Hypoxia+N-ADA+SR1	75,49±1,36 *	34,95±4,14 *,**
Hypoxia+N-ADA+SR2	73,96± 3,89 *	70,12±10,14 *
Hypoxia+N-ADA+Cpz	74,07±3,09 *	42,08±10,41 *,**

Table 1. Quantity of the living cells after hypoxia and after hypoxia under exposure to N-ADA and CB1-, CB2-, and TRPV1-receptor antagonists.

N-ADA-N-arachidonoyl dopamine (10 mcM), SR1-SR141716A (1mcM), SR2-SR144528 (1mcM), Cpz-capsazepine (1mcM).

*- $p < 0.05$ vs. intact group,

** - $p < 0.05$ vs. 1 DIV group. Kruskal-Wallis test.

network activity patterns (Table 1, Fig. 1). To reveal the involvement of some types of receptors in N-ADA neuroprotective effect, we used antagonists of CB1-R (SR141716A), CB2-R (SR144528), and TRPV1 (capsazepine) (Fig. 2A, B). CB1-R blockade counteracted neuroprotective effect of N-ADA at 1 DIV after hypoxia (Table 1) resulting in decreasing of burst quantity and increasing of spike quantity in burst, and at 7 DIV the network activity dramatically decreased. Exposure of cultures with N-ADA together with capsazepine also distinctly decreased neuromodulatory effects of cannabinoid. At the same time, CB2-R blockade did influence considerably neither viability nor bioelectrical properties of hypoxic neuronal network exposed to N-ADA.

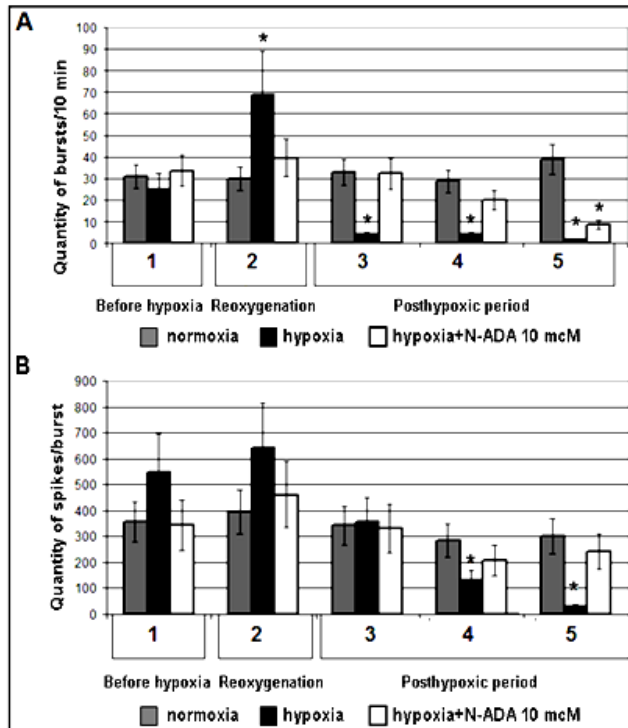


Fig. 1. Changes of patterns of neuronal network activity under exposure to hypoxia.

A – quantity of bursts/10 min; B – mean quantity of spikes/burst. Before hypoxia (1); 10 min reoxygenation (2); 2 hrs (3); 1 day (4) and 7 day (5) after hypoxia; *- $p < 0.05$ vs. 1 group, Kruskal-Wallis test

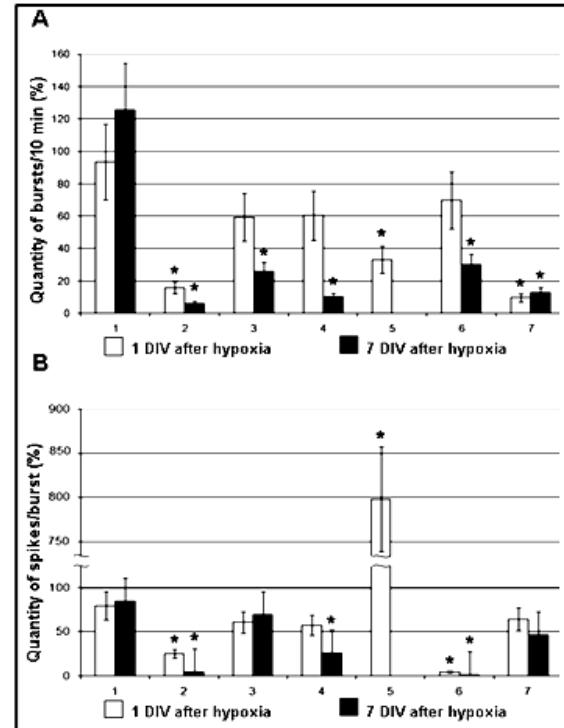


Fig. 2. Changes of patterns of posthypoxic neuronal network activity under exposure to N-ADA in the absence and in the presence of different receptor antagonists.

A – quantity of bursts/10 min; B – mean quantity of spikes/burst. Shame (1); hypoxia (2); hypoxia + N-ADA 10 mcM (3); hypoxia + N-ADA 2,5 mcM (4) hypoxia + N-ADA 2,5 mcM + SR141716A 1 mcM (5) hypoxia + N-ADA 2,5 mcM + SR144528 1 mcM (6) hypoxia + N-ADA 2,5 mcM + capsazepine 1 mcM (7); *- $p < 0.05$ vs. 1 group, Kruskal-Wallis test

In conclusion, our investigation of the mechanisms of N-ADA neuromodulatory effects on hippocampal neuronal network revealed not only the preferential protective role of CB1-Rs in posthypoxic period, but also involvement in these effects of TRPV1. Nevertheless, the common normalizing influence of N-ADA on viability and functional properties of cultured hippocampal neurons appears to be the consequence of fine adjustment of network activity as a result of N-ADA interaction with all three receptor types.

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ANTAGONISTS OF GABAERGIC RECEPTORS AS COGNITIVE ENHANCERS IN DOWN SYNDROME

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Abstract. Down syndrome (DS) is a developmental genetic disorder characterized by profound cognitive abnormalities. Mouse genetic models provide an opportunity to investigate cellular mechanisms of the cognitive impairment and to develop specific treatments improving cognition in DS. Here we overview recent findings of pharmacological treatments aimed at improving cognition in DS. Several classes of drugs reducing GABAergic inhibitory efficiency have been suggested as potential cognitive enhancers in DS models. The ability to rescue cognitive performance through treatments with drugs affecting GABAergic neurotransmission motivates studies to further explore the therapeutic potential of these compounds in people with DS.

Down syndrome (DS) is a developmental disorder caused by full or partial triplication of genes from chromosome 21 [1]. The presence of one extra copy of approximately 550 normal genes results in several notable clinical phenotypes including developmental delay and intellectual disability. The mechanisms of mental retardation in DS are not yet fully understood. Likewise, there are no approved effective specific treatments of intellectual disabilities in DS.

Mouse genetic models recapitulate major abnormalities of the brain specific for DS, thus providing the opportunity to better define the neurobiology of DS and to test the effects of potential treatments. The most widely used model is the Ts65Dn mouse in which the region extending from *Gabpa* to *Mx1* (~136 genes) is triplicated. This region contains genes responsible for many DS phenotypes including mental retardation. Ts65Dn mice exhibit a number of features typical of DS, including the impairment of learning and memory. Electrophysiological studies revealed severe impairment of hippocampal synaptic plasticity in Ts65Dn and other DS models. We demonstrated that impaired long-term potentiation (LTP) in the dentate gyrus (DG) of Ts65Dn mice could be fully restored by suppression of GABA_A receptors, suggesting that the excitatory/inhibitory balance may be altered in DS in favor of inhibition [2]. Subsequent histological, morphological, electrophysiological, and biochemical studies have provided solid experimental evidence for profound changes in inhibitory GABAergic circuits in mouse models of DS. Thus, it was shown that the immunoreactivity of several proteins associated with GABAergic synapses, including GABA_A receptor-associated protein (GABARAP), neuroligin 2, and vesicular GABA transporter (VGAT), was increased in the hippocampus of Ts65Dn mice [3]. These changes were accompanied by alterations in the microcircuitry of inhibitory inputs, with a significant increase in inputs to the necks of dendritic spines. Profound changes in inhibitory properties were observed in the hippocampus and neocortex of neonatal Ts65Dn mice, and the level of GAD-67 was increased in the Ts65Dn neocortex. Density of parvalbumin- and somatostatin-positive GABAergic neurons was increased in the hippocampus of juvenile Ts65Dn mice. We observed also that not only GABA_A, but also GABA_B receptor-mediated synaptic responses are increased in the Ts65Dn DG [4]. Increased efficiency of the inhibitory system suggests that one strategy to improve synaptic plasticity and, perhaps, cognition in DS would be through reduction of inhibition. Multiple recent studies confirmed efficiency of this strategy and showed that several classes of drugs restricting inhibitory neurotransmission may improve synaptic plasticity and cognition in DS.

Non-selective antagonists of the GABA_A receptors restored LTP in the hippocampus of Ts65Dn mice [2, 5] suggesting that treatments with such drugs may improve learning and memory. Indeed, subsequent studies showed that non-epileptic doses of the GABA_A receptor antagonist picrotoxin, pentylenetetrazol, and other GABA_A receptor antagonists improved performance of Ts65Dn mice in a number of hippocampus-mediate memory tasks [5-7]. The improvement of cognition in Ts65Dn mice following environmental enrichment was also linked to a decrease in GABAergic inhibition [8]. These findings provided first ‘proof of principle’ for the use of GABAergic inhibitors as specific cognitive enhancers in DS. However, profound pro-epileptic effects of such drugs may restrict their potential use in people. Therefore, other approaches aimed at reducing inhibition have been suggested.

Inverse agonists of alpha5 subunit-containing GABA_A receptors. It was proposed that use of subunit-selective drugs reducing efficiency of the GABA_A receptors may allow improvement of cognition

in DS without provoking epilepsy. Alpha5 subunit-containing GABA_A receptors are heavily expressed at extra-synaptic locations of pyramidal hippocampal neurons. It was demonstrated recently that selective inverse agonists of these receptors may improve synaptic plasticity and learning in Ts65Dn mice without exacerbating epilepsy [9]. One compound from this class of drugs is currently tested in a clinical trial by Hoffmann-La Roche Ltd (clinical identifier NCT01436955; <http://www.clinicaltrials.gov>), and the results of this trial should be reported in 2013.

Antagonists of GABA_B receptors. Another approach aimed at reducing inhibitory efficiency is blockade of metabotropic GABA_B receptors. We examined this approach using CGP55845, a high-affinity selective GABA_B receptor antagonist [10]. The drug fully restored LTP in Ts65Dn DG in hippocampal slices. Treatment with this drug of Ts65Dn mice significantly improved performance in tasks requiring long-term hippocampus-dependent memory. Thus, novel object recognition was fully restored in Ts65Dn mice by the treatments. Contextual fear conditioning was also improved, suggesting that the treatments reversed deficits in the hippocampus-dependent contextual memory. In contrast, increased locomotor activity and abnormal working memory were not affected in Ts65Dn mice. Thus, suppressing of post-synaptic GABA_B receptors may improve long-term memory in DS subjects.

Blockers of Kir3.2 potassium channels, effectors of the postsynaptic GABA_B receptors. Postsynaptic GABA_B receptors use as effectors inwardly-rectifying potassium channels containing Kir3.2 subunits. *Kcnj6*, the Kir3.2 encoding gene, is triplicated in DS and Ts65Dn mice. Owing to this genetic alteration, the level of the Kir3.2 protein is increased in Ts65Dn hippocampus by about 50%, resulting in enhanced signaling through the postsynaptic GABA_B receptors [4, 11]. Fluoxetine, which effectively blocks potassium currents through the Kir3.2 channels, restores LTP in Ts65Dn DG. Restoration of LTP was also observed in Ts65Dn mice containing only 2 copies of *Kcnj6* (Ts65Dn*Kcnj6*^{+/+} mice). Interestingly, it was recently shown that mice with 3 copies of *Kcnj6* exhibit deficits in hippocampus dependent learning and memory, as well as altered synaptic plasticity [12]. These findings suggest that blockers of Kir3.2 channels should be considered as potential cognitive enhancers in DS.

Thus, recent advances in understanding the neurobiology of DS lead to discoveries of new prospective treatments aimed at improving cognition in DS.

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A SIMULATION STUDY OF REVERBERATION IN DEVELOPING NEURONAL CULTURES

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Abstract. A simulation model based on detailed calcium synaptic dynamics is used to understand experimental observations of network reverberation during culture development. We find that there is an optimal set of parameters to produce reverberations suggesting that these parameters are related to network connectivity as observed in experiments. Furthermore, we find that the structure of the network is important in generating reverberations; scale free network cannot generate reverberation.

Spontaneous synchronized bursting is a fundamental feature of developing neuronal cultures and the characteristic of the bursts. The origin of the bursting and its time dependence are still unknown. The most interesting observation from the measurements of the spike time histogram of a synchronized burst by a multi-electrode-array system is that there are periodic structures as shown in Fig. 1a, suggesting that there are reverberations in the network. However, these reverberations can only be observed during a short range of days in vitro (DIV). The goal of our simulation study is to understand the mechanism of these reverberations and its relation with network structure.

Since it is known that reverberations can be generated by the Volman model [1, 2], our work is based on it. Figure 1b shows the Volman model used in our simulation. Briefly, X, Y, Z and S are the fractions of synaptic resources in the recovered, active and inactive states and super-inactive states respectively as shown in b). The total amount of synaptic resource is assumed to be a conserved quantity, hence $X + Y + Z + S = 1$. These states are related by some characteristic relaxation times as shown. Note that X can be released by either an action potential (stimulated release) or by residual calcium in the synapse (asynchronous release). These various states are related by some characteristic relaxation times (t_1 , t_2 , t_3 and t_4).

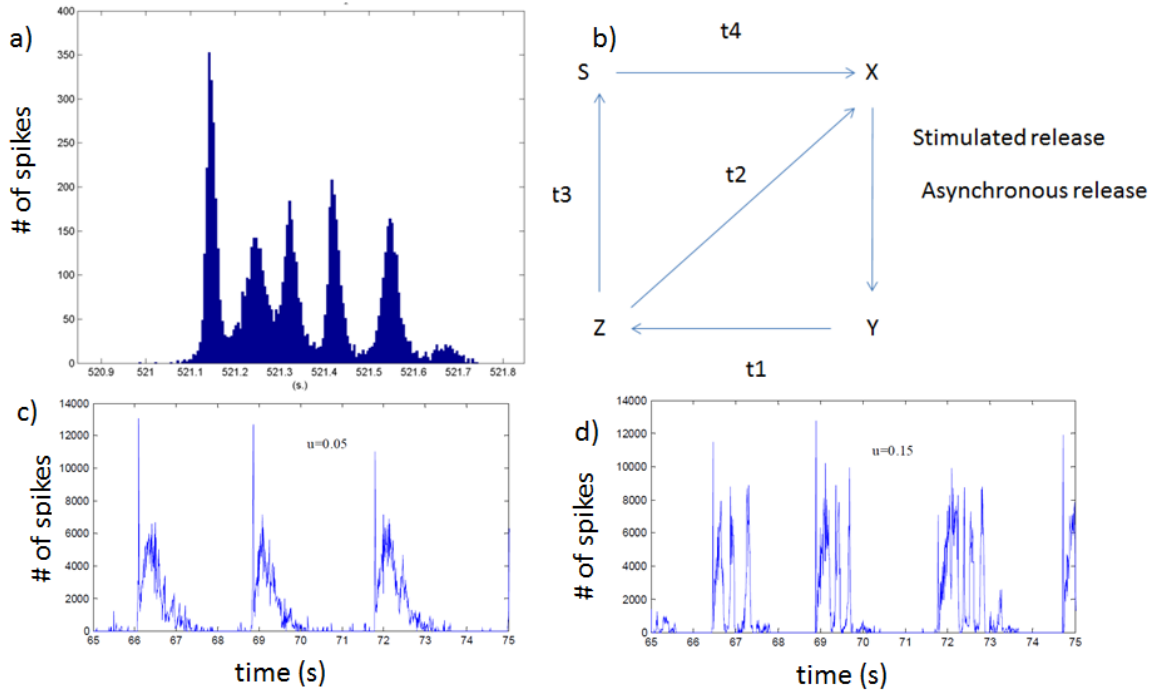


Fig. 1. a) Periodic structure in the spike histogram of a synchronized burst observed in experiment. b) The Volman model and c) Simulation result of spike histogram from the Volman model

One of the important parameters in the Volman model is the amount of stimulated release as controlled by the parameter u . In other words, u characterizes the strength of the synapse. When $u = 0$, there will be no synaptic release. Figures 1c and 1d are the results of the simulation of the Volman model with $u=0.05$ and 0.15 in a network of 100 elements. When compared with experiments, Fig. 1c and Fig. 1d resemble the spike histograms of experiments for early and late DIV respectively. This finding is consistent with our notion that synaptic strength increases during development.

The network used in the Volman model above is a random network. We are also interested in the relation between network structure and dynamics. In the simulations, we also make use of an algorithm developed by Morita [3] in which the structure of the network can be changed continuously from random to scale free. Figures 2a and 2c are the degree of distributions generated by this algorithm and Figs. 2b and 2d are the corresponding spike histograms generated by these networks. It can be seen that the scale free network (Fig. 2a) cannot generate the experimentally observed spike histogram while a random network is capable to do so.

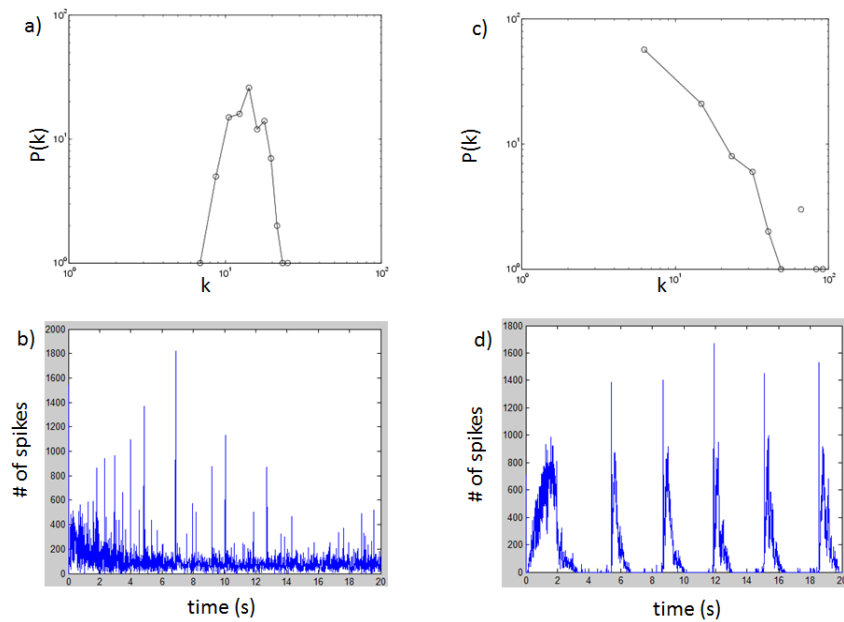


Fig. 2. Dynamics of different network topologies. a) Degree of distribution from a random network. b) Firing histogram of the network in a). c) Degree of distribution from a scale free network and d) firing histogram of the network in c)

Summary

1. Reverberations can be generated only when synapses are strong enough.
2. Reverberation the result of asynchronous release.
3. Scale free network in the Volman model cannot generate reverberations.

Acknowledgements

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DISENTANGLEMENT OF LOCAL FIELD POTENTIALS OPENS A WINDOW TO THE NETWORK DYNAMICS

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Abstract. Local field potentials (LFPs) recorded by electrode arrays provide spatiotemporal resolution sufficient to grasp electrical activity of neuronal assemblies associated with brain function. However, due to high complexity their use for studying ongoing information processing has been strongly limited. I provide a survey of recent results obtained in the rat hippocampus that enables separation of raw LFPs into so-called LFP-generators. This opens a window to description and quantification of the information transfer in neural networks.

LFPs as a potentially invaluable source of information

Brain nuclei continuously exchange information creating internal representations of the external reality. It has been proposed that, at least in part, synchronization of firing of multiple neurons is responsible for the information flow among cell assemblies. In this context ongoing LFPs offer an experimentally attractive tool for studying such flows with applications in the network dynamics, function, and brain-computer interfaces.

LFP is an extracellular voltage signal recorded with an intracranial electrode. It is one of the oldest measures of cerebral activity, yet much of its nature is unknown. The last decade has witnessed an explosive growth of experimental techniques that nowadays allow routine low-cost acquisition of tens or even hundreds of LFPs. Thus we've got a unique tool for sampling rapidly changing electrical activity with high spatial and temporal resolutions over big neuronal structures, like e.g. hippocampus. If we were able to decode LFPs and read the underlying information this would open a window into studying the integrative processes at level of neural populations.

One of the main challenges in this journey is the multisource complex nature of ongoing LFPs. They are raised by uneven summation of currents originated in different sites with different dynamics. Until recently the use of LFPs have been limited to a few specific events and oscillations. Recent progress in application of advanced mathematical methods to the analysis of LFPs shows their invaluable potential for studying function and dynamics of neural assemblies. Here I provide a survey on how LFPs can be employed for exploring the information transfer in the rat hippocampus. Special attention is given to the CA3-CA1 pathway (Fig. 1A), which is pivotal in encoding sequential memories.

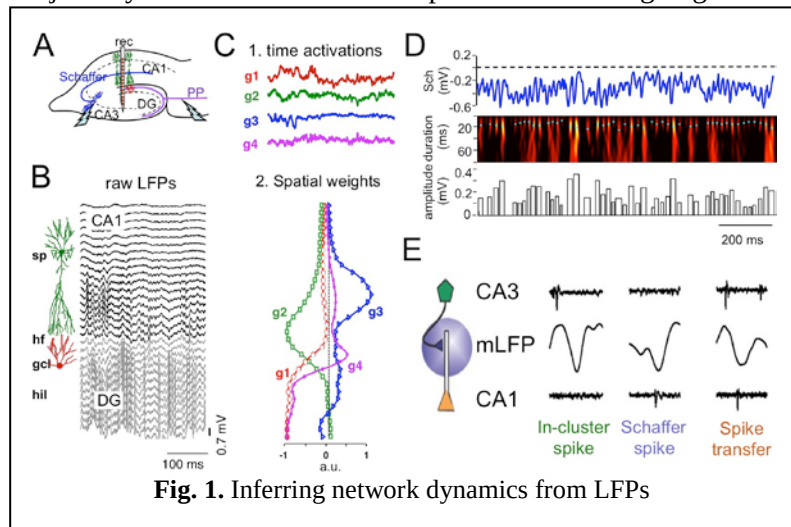


Fig. 1. Inferring network dynamics from LFPs

Disentanglement of LFPs: Experimental and theoretical framework

Figure 1B illustrates an example of raw LFPs recorded by a linear multi-electrode array (32 recording points) spanning CA1 and CA3 regions. One can appreciate the conspicuous presence of highly variable oscillatory patterns. These patterns enclose information on how principal cells integrate converging inputs from multiple neuronal populations. The oscillations can be handy for associating them to cognitive and behavioral tasks by blind correlations. However, this provides little insight on how the information processing occurs. Since signals from multiple neuronal sources overlap in time and space, looking at a raw LFP we cannot answer the question whether the oscillation is elicited by the activity of a local or distant source or by a mixture of several. The latter alternative is usually the case. Thus we have to identify the populations contributing to a given LFP as a necessary step to infer on its physiological and computational meaning.

Under reasonable assumptions the LFP, $V(t, x)$, created by synaptic current source density, $J(t, x)$, in a neural domain Ω with the conductivity σ can be described by the Poisson's equation:

$$-\sigma \nabla^2 V(t, x) = J(t, x), x \in \Omega \subset R^3$$

$J(t, x)$ is a compound current created by principal neurons driven by synaptic inputs coming from several afferent populations. Part of $J(t, x)$ corresponds to the input received by CA1 pyramidal cells from CA3. Then the problem is the following. Given experimental signals $V_j(t)$, $j = 1, 2, \dots, N$, how can we find synaptic currents corresponding exclusively to the Schaffer CA3-CA1 pathway $J_{Sch}(t, x)$?

In general, this inverse problem cannot be solved. However, in laminar brain structures (like the hippocampus or cerebral cortex) it admits a satisfactory solution. Multisite linear recordings (Figs. 1A and 1B) are well suited for this purpose. To disentangle the current sources we developed a method based on the independent component analysis [1,2]. It separates raw LFPs into components, so-called LFP-generators, responsible for different synaptic pathways converging on hippocampal neurons.

Each LFP-generator is defined by two elements: a curve of spatial weights and time course of the activity of the corresponding pathway (Fig. 1C). We found that only a few pathway-specific generators account for most of the LFP variance. Blue curves in Fig. 1C describe the excitatory Schaffer input from CA3 to CA1.

Schaffer LFP-generator as a marker of information flow in CA3-CA1 pathway

Once disentangled from raw LFPs, the Schaffer generator can be studied separately. We quantified spatial modules of synchronous activity related to overlapping of synaptic territories of CA3 clusters [3]. Then using the wavelet analysis [4] we identified and quantified patterns in the time course of the Schaffer generator.

Figure 3D shows that the Schaffer generator is made up of small wave-like LFP events appearing at gamma frequency. Traditionally, gamma activity in the hippocampus was assumed to be mainly inhibitory. Our findings show that gamma events in CA1 reflect in part compound synaptic currents generated by synchronous spiking in CA3 [5]. Using these events as a pivot we identified pairs of monosynaptically connected CA3 and CA1 neurons and studied ongoing spike transfer in the CA3-CA1 pathway (Fig. 1E).

We found that CA3 assemblies are not rigid constructs, as the neurons that comprise them may belong to different clusters and fire consecutively within them (Fig. 1E, in-cluster spikes). When an appropriate CA3 cluster fires, CA1 neuron generates an output spike (Schaffer spikes). Triple coincidences (CA3 spike, micro-LFP event, CA1 spike) describe effective spike transfer between two nodes in the network. Sorting CA1 spikes by their synaptic drive opens also a possibility to investigate plastic modulations in vivo [6]. Following induction of LTP by CA3 stimulation we found a moderate increase in the gamma power of raw LFPs in the CA1, but a strong increase in the power of the Schaffer generator.

In conclusion, the use of advanced mathematical methods for decoding information enclosed in LFPs provides significant insight on the network dynamics and function of brain nuclei.

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ATP-INDUCED CALCIUM SIGNALING IN RAT HIPPOCAMPAL CELLS

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Abstract. We investigate the spontaneous and ATP-induced changes in intracellular calcium in neurons and astrocytes CA3 field of rat hippocampus acute slices. The dependence between generation of $[Ca^{2+}]_i$ signals and the perfusion solution temperature, the voltage- Na^+ channel activity in the different age groups were revealed. It was shown in early ontogeny (P5) spontaneous calcium oscillations in neurons are regulated by mainly bioelectrical activity of a neural network. In the late neonatal period (P16) the addition of TTX insignificantly reduced the frequency of spontaneous calcium oscillations, while the addition of ATP increased the frequency of calcium events in background TTX, and inhibition of these events depended on the activity of some purinergic receptors and mechanosensitive channels.

Information processing in the brain is the result of the constant interaction between two cellular networks: the neuronal and the glial. Neural networks are integrated through electrical and chemical signals [1–3]. The transmission of information in the glial network is due to the diffusion of ions and molecules to the intercellular space. Although functional value of these alternative signaling pathways is largely unknown, it is increasingly clear that the cell-cell interactions in neuronal-glial networks are essential for normal functioning of the brain. In addition, the study of the functioning of the neuron-glial networks is necessary for understanding the formation of pathological events that determine the outcome of many neurological diseases [4, 5].

Purinergic receptors play a specific role in signaling in neuronal-glial networks, participating in all varieties of neuron-glial signaling, including Ca^{2+} - signaling as fundamental processes essential for the functioning of cells. Activation of purinergic receptors causes an increase in intracellular calcium ($[Ca^{2+}]_i$), which is an important messenger of the cellular response in the central nervous system (CNS). On glial cells and neurons expressed P2Y metabotropic and ionotropic P2X receptors are mobilizing $[Ca^{2+}]_i$. It is likely that different subtypes of purinergic receptors play a different role in the physiology and pathology of the cell. There is evidence that activation of P2 receptors is a key part in the launch of reactive changes in the cells, including expression of early genes, regulating the induction of intracellular kinase and cyclooxygenase-2, the synthesis of phospholipase A2, release of arachidonic acid and the production of prostaglandins and interleukins [6–9]. The relevance of the project is defined, first of all, study of the fundamental mechanisms of cell-cell interaction in neural networks of the hippocampus, in particular in the CA3 field in health and disease, and, secondly, the need to develop prevention and correction of post-ischemic disruption methods to the neural networks in the acute and chronic phase of circulatory ischemia after a stroke.

In this project we investigate the spontaneous and ATP-induced changes in intracellular calcium in neurons and astrocytes CA3 field of rat hippocampus acute slices, using a laser scanning confocal microscope Carl Zeiss LSM 510 DuoScan (Germany). Entries of fluorescence kinetics were carried out in full frame (field of view of 400×400 μm), with a digital resolution of 256×256 pixels and a scan rate of 1 Hz. Fluorescence indicators were recorded in the range 500–530 nm (Oregon Green 488 BAPTA-1 AM) and 650–710 nm (Sulforhodamine 101). The fluorescence intensity ($\Delta F/F$) shows the dependence of the concentration of $[Ca^{2+}]_i$ on time, indicating metabolic activity of cells.

Spontaneous $[Ca^{2+}]_i$ signals were observed in neurons and astrocytes. Neuron $[Ca^{2+}]_i$ signals are associated with the release of neurotransmitters, synaptic plasticity and electrical excitability. Astrocytes electrically are nonexcitable, therefore $[Ca^{2+}]_i$ signals occur in response to chemical or mechanical stimuli. As a result of the study, the dependence between generation of $[Ca^{2+}]_i$ signals and the perfusion solution temperature, the voltage- Na^+ channel activity in the different age groups (P5–8, P14–16, P21–25) were revealed. The studies showed a reduction in the duration and an increase in the frequency of $[Ca^{2+}]_i$ events in pyramidal neurons and interneurons with perfusion solution at 35°C in comparison with those at 24°C, due to increased metabolic activity of cells. The frequency and duration of the $[Ca^{2+}]_i$ events in astrocytes did not change significantly. We examine the role of various purinergic receptors in ATP-mediated interactions between neurons and glial cells. It was shown that in early ontogeny (P5) spontaneous calcium oscillations in neurons are regulated by mainly bioelectrical activity of a neural network (Fig. 1 A.).

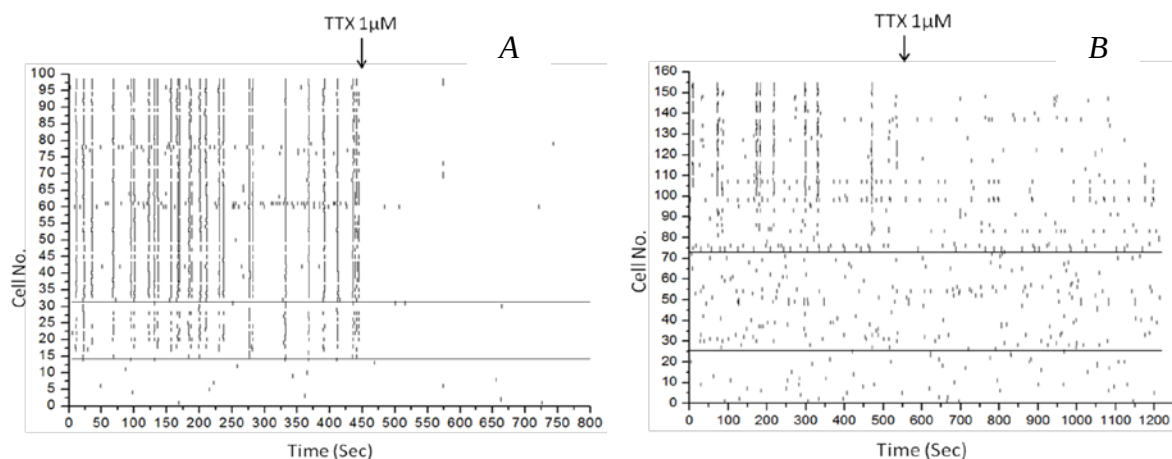


Fig. 1. Raster plots of spontaneous Ca^{2+} activity recorded from cells CA3 field of rat hippocampus. Adding TTX ($1\mu\text{M}$) shown with an arrow at the top (↓). A. Cells CA3 field of rat hippocampus P(5). Cells 1–13 correspond to astrocytes, 14–31 correspond to interneurons and 31–98 correspond to pyramidal neurons. B. Cells CA3 field of rat hippocampus (P16) Cells 1–26 correspond to astrocytes, 27–73 correspond to interneurons and 74–154 correspond to pyramidal neurons.

In the late neonatal period (P16) the addition of TTX insignificantly reduced the frequency of spontaneous calcium oscillations (fig. 1B), while the addition of ATP increased the frequency of calcium events in background TTX, and inhibition of these events depended on the activity of some purinergic receptors (ionotropic and metabotropic) and mechanosensitive channels. The scientific significance of the project is to provide new fundamental knowledge about the role of electrical and chemical components of calcium signaling in hippocampus neuronal-glial networks of different age groups.

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ANTIHYPOXIC EFFECT OF N-ARACHIDONOYL DOPAMINE IN THE HIPPOCAMPAL CULTURE NEURON NETWORK

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Abstract. Antihypoxic effect of the endocannabinoid N-arachidonoyldopamine (N-ADA) on spontaneous calcium activity of dissociated hippocampal cultures after hypoxic damage is studied. Inhibition of spontaneous calcium activity of neurons after acute hypoxia/reoxygenation was shown. The addition to the culture medium of N-ADA during hypoxia and the first day of posthypoxic period reduces the damaging effect on the neuronal calcium functional activity.

The endogenous cannabinoid system plays an important role in the regulation of brain functions, including neural excitability, glia function and immune responses. There are numerous studies showing that cannabinoid and vanilloid receptor ligands exert neuroprotective properties in different models of brain injury [1-4]. N-arachidonoyldopamine (N-ADA) was recently described as cannabinoid (CB1) and transient receptor potential vanilloid-type-1 (TRPV1) receptor agonist [5, 6]. The molecular mechanisms underlying the neuroprotective properties of endocannabinoids are multiple. Endocannabinoids can reduce glutamate release, calcium influx and/or inflammation, improvement of glucose utilization, or alternatively the production of ketone bodies [3, 4, 7, 8]. Also, there are other mechanisms. Hypoxia is a pivotal factor of neuron injury during ischaemia disorders, for example, stroke, and endocannabinoids can realize their protective effects during hypoxia through cannabinoid CB1/CB2 receptor-dependent and -independent mechanisms.

We studied the effect of N-ADA on the neural network activity in dissociated hippocampal cultures during hypoxia. We investigated patterns of spontaneous calcium oscillations in mice dissociated hippocampal cultures *in vitro*, using fluorescent calcium dye Oregon Green 488 BAPTA-1 AM and Zeiss LSM510 NLO Duoscan confocal microscope for recording of time-series Oregon Green fluorescence intensity. Modeling of hypoxia was performed on 21st day *in vitro* by replacing the normoxic culture medium by a medium with low oxygen for 10 minutes. In experimental groups N-ADA (2.5 μ M, 6 μ M and 10 μ M) was added into culture medium during acute hypoxia and by first day of posthypoxic period. We found that spontaneous Ca^{2+} activity is a common property of most neurons and astrocytes that was lost in response to a hypoxia. Ca^{2+} transients were almost completely absented on the third day after hypoxic action in 50 % of cultures. By the seventh day after hypoxia some cultures demonstrated pathological activity (abnormal long calcium "superoscillations") and in some cultures activity reduced completely. It was shown that N-ADA (2.5–10 μ M/ml) dose-dependently protected neurons and normalized activity dissociated cultures of hippocampus in the distant posthypoxic period (Fig. 1).

To identify the mechanism of N-ADA mediated neuroprotection effect, we used SR 141716A like an antagonist of the endocannabinoid receptor 1, SR 141716A like endocannabinoid receptor 2 antagonist and capsazepine like TRPV1 antagonist. Endocannabinoid receptor antagonists did not significantly modulate N-ADA neuroprotection while capsazepine reduced the neuroprotective effect in our experiment. Finally, endocannabinoids may interact with non-cannabinoid, non-TRPV1 receptors and channels, and, although the full physiological relevance of such interactions is yet to be established, the "antihypoxic effect" of this lipophilic molecule can activate potential ways to affect Ca^{2+} signaling. Here we discuss the effects of endocannabinoids on spontaneous Ca^{2+} transients and their potential protective consequences.

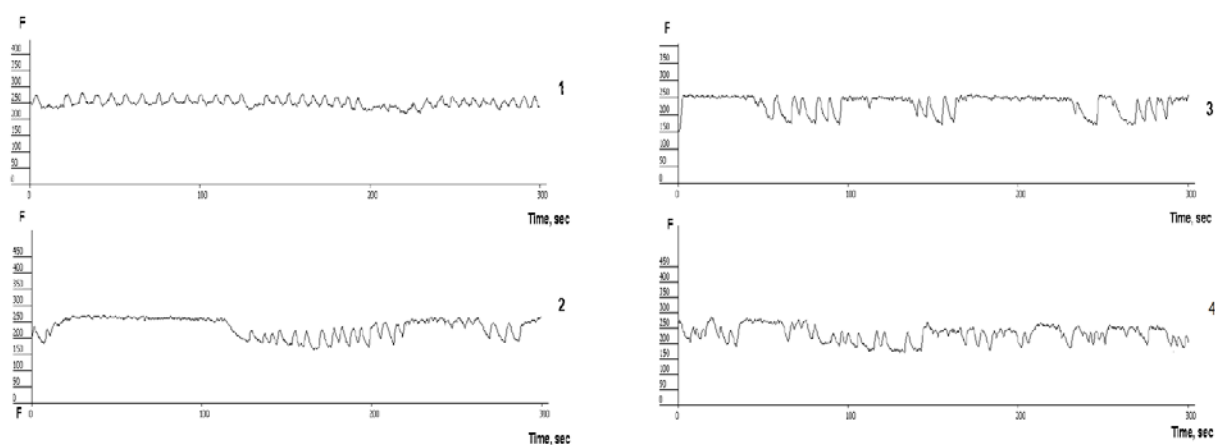


Fig. 1. Examples of Ca^{2+} transients: 1 – Shame; 2 – After hypoxia, 3 – N-ADA 2,5 μM , 4 – N-ADA 10 μM

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MICROELECTRODE ARRAYS AND Ca^{2+} IMAGING IN COMBINATION WITH IN VITRO MODEL OF STROKE AS A TOOL TO INVESTIGATE PATHOLOGICAL CHANGES IN NETWORK ACTIVITY

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Abstract. An application of microelectrode arrays (MEAs) technology to study the mechanisms underlying pathological processes evoked by stroke or brain ischemia was shown. In this review, the use of dissociated hippocampus culture and slides from rodents is discussed to study rhythmic bioelectrical activity and spontaneous Ca^{2+} transients in neurons and astrocytes. In particular, network analysis revealed that most neurons formed correlated networks, which were synchronous with both astrocytes and neurons in some pathological conditions. These pathological processes which evolve via different cell mechanisms are discussed as a paradigm to apply MEA recording for detailed mapping of the functional damage and its time-dependent evolution.

Microelectrode arrays (MEAs) represent an important tool to study the basic characteristics of neuronal networks that provide learning, memory and other kinds of plasticity in physiological conditions [1]. Fundamental properties of this neuronal rhythmicity like burst origin, propagation, coordination, and resilience can, thus, be investigated at multiple sites within the certain morphology of the circuits [2]. New technologies can be useful for studying and eventually developing new strategies to treat diseases of the brain, which all represent an important burden to society and which, too often, remain incurable [3].

Spontaneous neuronal activity is essential to neural development. However, cultured astrocytes and, more recently, astrocytes in situ and in vivo are now known to exhibit spontaneous Ca^{2+} transients [4]. Here we used Ca^{2+} imaging of astrocytes and neurons from mice primary hippocampal culture and slides for the simultaneous monitoring of $[\text{Ca}^{2+}]_i$ changes in large numbers of cells.

We found that spontaneous activity is a common property of astrocytes that is changed in response to a lesion. Moreover, network analysis revealed that most hippocampal culture astrocytes formed correlated networks, which were synchronous with neurons. It was shown that such factors of ischemia as hypoxia and glucose deprivation changed spontaneous Ca^{2+} transients in neurons and astrocytes. These results show that spontaneous activity in astrocytes and neurons is patterned into correlated neuronal/astrocytic networks in which neuronal activity regulates the network properties of astrocytes.

Here we discuss the effects of short-term hypoxia and glucose deprivation like precondition for future long-term hypoxia and glucose deprivation. Interestingly enough hypoxia/reoxygenation causes an increase in the number of active neurons and astrocytes and in their network synchrony with subsequent loss of this activity. It is now becoming clearer that critical changes in neuronal bioenergetics and oxidative stress during reoxygenation are important players in the neuronal death associated with stroke. The increase of active neurons and astrocytes depend on excitotoxicity, a phenomenon that occurs when the N-methyl-D-aspartate (NMDA) glutamate receptor subtype (NMDAR) is overactivated. Together, these models demonstrate simplification of network structure but saving of hubs in correlation graph analysis.

Furthermore, activation of endogenous modulators endocannabinoids and endovanilloids, and neurotrophic factors BDNF and GDNF we considered like neuroprotection against ischemia injury in vitro. We found that lack of neuronal network overactivity after reoxygenation or re-glucose under the drug treatment is predictor measure of neuroprotective effectiveness.

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LEARNING AND ADAPTATION IN DISSOCIATED NEURAL CULTURES GROWN ON MULTIELECTRODE ARRAYS

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Abstract. Learning in neuronal networks can be investigated using dissociated cultures on multielectrode arrays supplied with appropriate closed-loop stimulation. In previous studies it was shown that probability of certain spiking response to a single electrical stimulus can be significantly increased. Such condition was achieved using a protocol with continuous stimulation and reinforcement condition in the closed-loop. However, such protocol was applied to the neurons with relatively weak connections. We introduced enhanced protocol of learning with adaptive reinforcement condition.

Cell cultures were prepared from the hippocampus of C57Bl6 mice embryos at 18-th prenatal day (E18) following standard procedures. The cultures were plated on 64-electrode arrays (Alpha MED Science, Japan). The final density of cell culture was about 15,000-20,000 cells/mm² (Fig. 1A). Experiments were performed when neuronal networks were 3-6 weeks *in vitro* that permitted their functional and structural maturation (see Pimashkin et al. 2013 for details).

Stimulation consisted of two stages – control stimulation and training stimulation of a randomly chosen electrode. Trains of biphasic rectangular voltage pulses (600 mV and 300 μ s per phase) at low-frequency (0.05-0.06 Hz) were applied for 8 hours in cyclic (10 min stimulation followed by 5 min no stimulation) control stimulation. Each stimulus evoked reverberating spiking activity in the network (Fig. 1B). The training stimulation consisted of real-time evaluation of the response measure R/S (response to stimulus) on the selected electrode as a number of spikes in 40-80 ms after stimulus artifact averaged over 10 preceding responses. The stimulation was performed by continuous acquisition of the stimulus response. The reinforcement condition was defined as follows. If the response exceeds the estimated threshold the stimulation stops for 5 min.

First we analyzed the characteristics of the responses in the control stimulation. Particular electrodes for training stimulation were selected among the electrodes with significantly higher responses, where average R/S = 0.1-10 versus $R/S \leq 0.1$ in the previous studies from Shahaf et al, 2001; Li et al, 2007; Stegenga et al, 2009; Le Feber et al, 2010. The fraction of such highly responded neurons within electrodes was 58.16% (n=10 cultures) versus 31.13% of the low active as in previous studies. To apply the closed-loop protocol to such highly active neurons we introduced the adaptive threshold estimation. After the control stimulation was performed, the distribution of all responses R/S appeared after each stimulus was estimated. Then the value of the 90-th percentile of the distribution (rare R/S values with 10% probability) was set as R/S threshold and was defined by R/S threshold estimation parameter $R/S_{Thr\%} = 10\%$. Typically it was in the range 0.2-12.

We found that without closed-loop in control condition the appearance of the estimated R/S threshold was stable and time to achieve it after each stimulation cycle ($T_{R/S}$) was relatively high, typically 400 sec and cycle duration - 600 sec (Fig. 1C).

In these closed-loop conditions the stimulation was turned off for 5 min till the next cycle when the RS of the response reached R/S threshold. We found that the adaptation time ($T_{R/S}$) decreased (Fig. 1 D). We characterized the decrease of adaptation time ($T_{R/S}$) as the ratio $K(T_{R/S})$ estimated as mean $T_{R/S}$ in the last 10 cycles divided to the mean of the $T_{R/S}$ in the first 10 cycles. The decrease of $T_{R/S}$ during the stimulation cycles ($K(T_{R/S}) < 0.5$) was then treated as successful learning for the neurons on the selected electrode to generate a desired response on the stimulation electrode. Statistics of successful trials was about 50% which was comparable to the earlier studies (Shahaf et al, 2001; Le Feber et al, 2010). We performed training stimulation using R/S threshold estimation based on various $R/S_{Thr\%}$ - 5%, 10%, 15% and 20%. Thus we analyzed how probability of the rare responses in reinforcement condition affects learning. We found that only using $R/S_{Thr\%}=15\%$ the protocol application led to successful learning (Fig. 1 E).

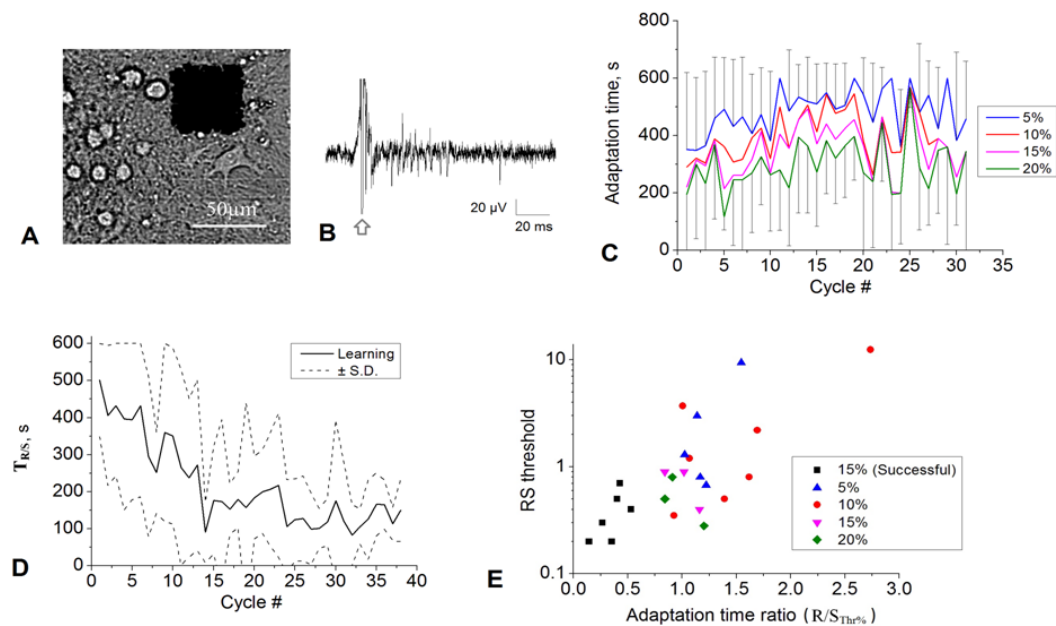


Fig. 1. (A) Cultured hippocampal cells grown on multielectrode array with 64 square electrodes. (B) Post-stimulus response recorded from single electrode. (C) Learning curves calculated for control experiments using different threshold estimation parameter $R/S_{Thr\%}$ ($n=14$). (D) Average learning curve (6 experiments, 6 cultures). Dashed curves illustrate the standard deviation. (E) The R/S threshold values for successful (black markers) and failed (colored markers) learning experiments. The colored markers correspond to the use of different values of the threshold estimation parameter, $R/S_{Thr\%}=5\%$, 10% , 15% and 20% of R/S

In summary, we proposed an adaptive closed-loop stimulation protocol capable to achieve learning even for the highly respondent electrodes. We introduced an adaptive reinforcement condition accounting for the response variability in control stimulation. It significantly enhanced the learning protocol to a large number of responding electrodes independent of their basal response levels. The results show the successful rate similar to the previous studies.

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THE EFFECT OF THE BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) AND K252A ON THE SPONTANEOUS NEURAL NETWORK ACTIVITY OF PRIMARY DISSOCIATED HIPPOCAMPAL CULTURES DURING HYPOXIA IN VITRO

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Abstract. We investigated the effect of the preventive application of BDNF and the high-affinity neurotrophin receptor antagonist k252a on the survival and main parameters of spontaneous bioelectrical activity of dissociated hippocampal cultures in the acute hypoxia and in the posthypoxic period. Our experiments revealed preventive application of BDNF before hypoxia reduced the negative effect of oxygen deficiency by preservation of network burst activity and viability of cells. The addition of BDNF with k252a was similar but less effective than application of the neurotrophin only. We suggest the antihypoxic properties of BDNF can be supported not only by TrkB receptor mechanism.

Hypoxia is considered as one of the main factors of brain cell damage in ischemia and a number of other pathologies. Due to alteration in oxygen supply there are significant changes of synaptic transmission processes related to cells death and destruction of neuronal networks in the brain [1]. Nowadays there is an active search of substance able to protect cells from negative effect of hypoxia. Brain-derived neurotrophic factor (BDNF) is an important member of the neurotrophin family of growth factors that may be considered as one of the potential substances to control cell metabolic rate under hypoxemia [2-5]. The main functions performed by BDNF are determined by its interaction with the tropomyosin-related kinase B (TrkB) receptor, which launches important signaling mechanisms involved in different processes occurring in neurons [6,7]. However, a question of the certain signaling mechanism of BDNF in the oxidative process control in mature brain remains unclear.

The aim of the investigation was to study the effect of BDNF and the inhibitor of the TrkB receptor k252a on the survival and main parameters of spontaneous bioelectrical activity of dissociated hippocampal cultures in short-term hypoxia and within 7 days of the posthypoxic period.

Dissociated hippocampal cells were taken from the brain of mice embryos (E18) and cultured by previously developed protocol more than 40 days in vitro (DIV) on the multielectrode arrays (MEA60, MEA6-well, Multichannel Systems, Germany) [8]. Culture viability was maintained under constant conditions of 35.5°C, 5%CO₂ at saturating humidity in CO₂-incubator (Sanyo, Japan). Starting density of cell culture on the matrix was 17000 cells per mm². Modeling of hypoxia was performed on 33rd DIV by replacing the normoxic culture medium by a medium with low oxygen for 10 minutes. In experimental groups BDNF (1 ng/ml) and BDNF in combination with k252a (150nM) was added into culture medium 20 minutes before acute hypoxia. Main characteristics of spontaneous neural network activity were investigated: a number of small bursts; a number of spikes per burst. The criterion of a burst was the presence of spikes on minimum four different electrodes of the arrays, the interval being not above 100ms. All signal analysis and statistics were performed using customized software MC Rack (Multichannel Systems, Germany) and Matlab. There was viability evaluation of dissociated hippocampal cells by calculation of percentage ratio between the number of dead cells stained by propidium iodide (Sigma, USA) and total number of cells stained by bisBensimide (Sigma, USA) on the 3rd and 7th days after hypoxia.

The carried out experiments revealed 10-minute normobaric hypoxia to cause irreversible changes in spontaneous bioelectrical activity and increase the number of dead cells during 3 days after injury. Preventive application of BDNF (1 ng/ml) 20 min before hypoxia reduced negative effect of oxygen deficiency by partial preservation of network burst activity during injury, and the normalization of the parameters of spontaneous bioelectrical activity in the post-hypoxic period. Moreover, the number of dead cells was statistically significant ($p < 0.05$) lower than in the control cultures. The effect of application of BDNF (1 ng/ml) in combination with k252a (150nM) was shown to be similar but less effective than BDNF addition only. Therefore, we suppose the antihypoxic properties of BDNF are associated not only with the interaction with TrkB receptor but also can be supported by another signaling mechanism.

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CONFORMATIONAL DYNAMICS OF DNA: BIOLOGICAL MANIFESTATIONS AND PHYSICAL APPROACH

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Abstract. Hairpin or cruciform DNA structures can generate micro RNAs (miRs). Their dysregulation provokes neurodegenerative and other diseases. Since the first example of miRs regulated genes is LIMK1, the key enzyme of actin remodeling, we have sequenced the *Drosophila dlimk1* gene. Intron I of the mutant *agn^{ts3}* carries a 28 bp insertion partially homologous to dme-miR-1006, is nucleosome-free, participates in ectopic chromosomal pairing and demonstrates unusual DNA conformational dynamics. These findings are discussed in terms of molecular genetics and physics of condensed matter at phase transformations.

Neurodegenerative diseases, such as Huntington's, Parkinson's and Alzheimer's diseases are envisioned as cofillinopathies because of LIMK1 dysfunction leading, in particular, to cofilin-actin coated amyloid plaques. LIMK1 is a key enzyme of actin remodeling (remodeling small GTPases of Rho family – LIMK1 – cofilin – actin). LIMK1 phosphorylates cofilin and thereby affects actin filament dynamics leading to dendrite spine reorganization required for synaptic plasticity and learning/memory. On the other hand, these diseases are presumed to result from dysregulation of micro RNAs (miRs), LIMK1 being the first documented target of miRs regulation [1-2]. It is believed, that MiRs themselves are generated by hairpin or cruciform structures of DNA attained by promoters, sites of transposon insertions, introns of a gene, etc [3]. DNA cruciforms are fundamentally important for a wide range of biological processes, including replication, regulation of gene expression, nucleosome structure and recombination evolution and development of diseases. Moreover, single nucleotide polymorphisms and/or insertion/deletion mutations at inverted repeats located in promoter sites can also influence cruciform formation, which might be manifested through altered gene regulation [4]. Having these in mind, we have launched a multi-facet biological and physical study of the *Drosophila* LIMK1 gene (*dlimk1*) with a special attention to biological consequences of DNA secondary structure and DNA conformational dynamics.

The *limk1* gene is harbored by the *Drosophila agnostic* locus previously found in our mutational screen in 11B region of the X chromosome with specific architecture: the enrichment in A/T-tracts predisposes both to transposon insertions and to spontaneous gene rearrangements in different natural populations. Among these are the wild type *Drosophila* strain *Canton-S* (CS) and EMS-induced mutant *agn^{ts3}*. We have sequenced the *limk1* gene (GeneBank: *Dlimk1_CantonS* – JX987486; *Dlimk1_agnostic3* – JX987487). Compared to the genome sequence, *LIMK1* of the mutant carries the single- and multi-nucleotide polymorphisms, including base changes, insertions and deletions, mainly within introns, the most prominent being the insertion of 28 bp in I intron partially homologous to dme-miR-1006.

It is known, that the genomic sequence is highly predictive of the in vivo nucleosome organization, even across new nucleosome-bound sequences isolated from fly and human, and thereby imposes quite rigid rules of transcriptional regulation [5]. Therefore, we used NuPoP software: for the analysis of probable nucleosome sites in the genomic DNA of the *dlimk1* (Flybase; extended gene; 0-11776 bp.) and the abovementioned *agn^{ts3}* genomic sequence. The obtained results indicate that *dlimk1* is not constitutive and is regulated by nucleosome remodeling in promoter regions. Two nucleosomes are depleted from the 1st fragment of intron 1 (about 270 bp, 2536-2806 bp) in *agn^{ts3}*. Also, nucleosome-depleted is the other fragment of intron 1 (100 bp, 3300-3400 bp) which carry a A/T-rich 28bp insertion capable of miR formation. This enables the region to manifest an unusual conformational dynamics confirmed both by our bio-informatic analysis (RNAstructure software), and by physical methods of Brillouin light scattering.

Since nucleosome-depletion opens a possibility for interaction of a certain DNA region and its flanking DNA with any protein, as well as with any region of the same or different chromosomes, namely imposes the rules of chromosome pairing, all strains were subjected to a study of ectopic (non-homologous) pairing between the 11B region of the X-chromosome (*dlimk1*) and all other *Drosophila* chromosomes. The strain-specific regions of ectopic chromosomal contacts were plotted together with

positions of target genes for dme-miR-1006. This indicated a strain- specific pattern of chromatin modifications. Moreover, this pattern in *Canton-S* and *agn^{ts3}* suffered marked alterations upon temperature regimes shifts of strain maintenance. This dramatically different pattern of ectopic pairing of miR target genes indicated a new constellation of simultaneous transcriptional regulation.

From the viewpoint of physics, conformational dynamics is an intrinsic property of DNA that changes when one conformation transforms into another under varying external conditions, such as temperature, pressure, etc. It results from changes in the spectrum of inborn vibrations of a molecule. The low-frequency region of the vibrational spectrum of DNA (from 1 to 1000 GHz) is of highest importance, because the changes in vibrations caused by changes in the spatial organization of DNA at conformational transitions are manifested in this frequency region. The most informative technique suitable for studying the low-frequency conformational dynamics is Brillouin light scattering which covers the frequency in the abovementioned range and allows obtaining direct evidences of the processes associated with both the emergence of local structures of DNA and its melting. Theoretical models describe mainly DNA transformations at melting in terms of the spiral-coil transition and do not consider changes in the spatial organization of DNA accompanying the emergence of local structures, such as hairpins, crosses, etc. They can drastically change the low-frequency dynamics of DNA and require a separate consideration in both mathematical modeling and experimental studies. However, these studies are scarce and contradictory. This concerns both the well known melting of DNA and the poorly studied conformational transformations accompanying formation of local structures. The dynamics of DNA at the A-B phase transition is better understood. Inelastic light scattering experiments which revealed a soft mode at the A-B transition are well known [6, 7]. However, these investigations dating back to the 1980s were not continued despite their importance

We employed the Brillouin light scattering technique to investigate the low-frequency dynamics at DNA melting in two different samples of DNA with known nucleotide sequence from the I intron of *dlimk1* gene, one being from the mutant *agn^{ts3}* (locus *agnostic*, 120 bp long), the other from the wild type strain *Canton-S* (CS, 100 bp), DNA concentration 485 mkg/ml. Both DNA samples were obtained from genomic DNA by a PCR. Conformational dynamics manifested itself in the light scattering spectra well below the DNA melting temperature. The calculations performed showed temperature-dependent variations in local DNA structures which were specific either to the wild type, or to the mutant DNA. This was confirmed by the analysis of the temperature evolution of light scattering spectra for the mutant and the wild-type.

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SPATIOTEMPORAL PROPERTIES OF CALCIUM DYNAMICS IN HIPPOCAMPAL ASTROCYTES

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Abstract. Astrocytes produce a complex repertoire of Ca^{2+} events that coordinate their major functions. However, the canonical cellular mechanism for Ca^{2+} integration in astrocytes is unknown. Our analysis of the spatiotemporal dynamics of Ca^{2+} signaling in single hippocampal astrocytes in culture and in brain slices demonstrated that Ca^{2+} event distribution can be described by a *power law*, a fingerprint of scale-free systems. We further show that the exponent of the power law (α) decreases following the activation of metabotropic glutamate receptors (mGluRs). This principle of integration of Ca^{2+} events in astrocytes provides a new insight into neuro-glia communication.

Astrocytes are electrically non-excitable cells that form a support network for neurons in the brain. Astrocyte-neuron communication plays an important role in the operation of neuronal networks [1, 2]. In response to neuronal activity, astrocytes can release a number of neuroactive substances, or gliotransmitters that alter synaptic transmission and neuronal excitability. Gliotransmitter release is often sensitive to, or directly triggered by, cytosolic Ca^{2+} events in astrocytes [2]. Recently, we reported that the level of Ca^{2+} activity in astrocytes determines synaptic coverage by the astrocytic processes and perisynaptic glutamate uptake [3]. Thus, Ca^{2+} dynamics comprises a major cellular signaling mechanism in astrocytes. The principle of Ca^{2+} events integration in astrocytes, however, is unknown.

The astrocytes were co-cultured with neurons, which allowed them to develop processes, and transfected with a genetically encoded Ca^{2+} sensor, GCaMP2. The astrocytes exhibited a variety of Ca^{2+} events with different spreads and durations. In contrast to traditional region-of-interest (ROI)-based imaging, we identified the areas occupied by individual Ca^{2+} events in each frame as regions of interconnected pixels exceeding the threshold of fluorescence change, $\Delta F/F$. The maximal projection area ($S_{\max}$) and duration were obtained for each event. The probability density of $S_{\max}$ and the duration of Ca^{2+} events were skewed and, when plotted on log-log scale, showed linear dependence suggesting that they satisfied a *power law* ($\text{Pr}(x) \propto x^{-\alpha}$, where x is $S_{\max}$ or duration. The exponent of the power law (α) was $\alpha = 2.58 \pm 0.07$ for $S_{\max}$.

The power law distribution suggests that $S_{\max}$ and duration of Ca^{2+} events may have scale invariant properties [4, 5], i.e. the power law distribution of these parameters obtained in the entire cell should be also observed in subcellular regions. Scale invariance suggests that the power law distribution of Ca^{2+} events can be also studied in a single focal plane of astrocytes in brain slices despite such events representing only a part of the overall Ca^{2+} activity. We then monitored astrocytic Ca^{2+} dynamics with two-photon imaging in adult hippocampal slices prepared from mice with astrocyte-specific GCaMP2 expression. The distributions of $S_{\max}$ and durations followed a power law in 12 and 10 of 13 recorded CA1 *stratum radiatum* astrocytes in slices, respectively ($S_{\max}$: $\alpha = 2.68 \pm 0.23$, duration: $\alpha = 3.01 \pm 0.14$). Then we imaged Ca^{2+} events in the baseline conditions and in the response to low frequency (0.2 Hz) stimulation of the Schaffer collaterals. The $S_{\max}$ distribution of Ca^{2+} events became tail 'heavier' during the stimulation, which was reflected by a decrease of the power law exponent. In the presence of 500 μM (S)- α -methyl-4-carboxyphenylglycine (MCPG; an mGluR antagonist) low frequency stimulation did not produce a decrease in the power law exponent suggesting that the change in the $S_{\max}$ distribution was mediated by mGluRs.

Thus, we describe a novel power-law based principle of spatiotemporal integration of Ca^{2+} events in hippocampal astrocytes, and regulation of this integration by neuronal activity.

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A SIMPLE NETWORK MODEL OF EPILEPTIFORM ACTIVITY IN DISSOCIATED NEURONAL CULTURES

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Abstract. Dissociated cultures of neurons are known to produce epileptiform patterns of spiking activity under certain conditions. These patterns also called “superbursts” consist of multiple highly synchronized population discharges, i.e. bursts, either following each other with relatively short interburst intervals or stuck together without clear gaps between them. We present a mathematical description of epileptiform superburst generation in a model of dissociated hippocampal culture. The model is a recurrent network of spiking neurons interconnected with synapses undergoing short-term synaptic plasticity. The dynamic mechanisms of normal bursting and pathological superbursting as well as scenarios of transitions between them are investigated numerically and analytically.

Enzymatic digestion of extracellular matrix in neuronal networks composed of dissociated cultured neurons was recently proposed as a new *in vitro* experimental model of epileptogenesis [1]. The experimental model uses a complex pattern called “superburst” as an indicator of epileptiform activity. Epileptiform superburst contains several normal population bursts with relatively small number of spikes per each and with much shorter interburst intervals compared to the normal bursting activity. Mechanisms of such superburst formation were investigated experimentally using various pharmacological applications to reveal the main molecular and cellular processes triggering transitions from normal to pathological activity. However, dynamic principles of such transitions and their key properties and conditions at the network level still remain uncovered. To bridge the gap between the molecular, cellular and the network levels of understanding the epileptogenesis, a mathematical description of neuronal network signaling in normal and pathological conditions should be considered.

We propose a spiking model of epileptiform activity in dissociated cultures of neurons. The model is based on Izhikevich neurons [2] fine-tuned to reproduce spiking behaviour of basic types of hippocampal neuronal cells. The neurons are synaptically interconnected into a recurrent network and the connections are modelled using Markram-Tsodyks synapses [3] tuned to show short-term depression and facilitation depending on the source and the target neuron types. Synaptic currents are simulated to mimic kinetics of four receptor types, i.e. AMPA, NMDA, GABAA and GABAB receptors. It was previously shown that in normal conditions the mechanisms of burst generation are concerned with avalanche-like propagation of activity from small population of excited neurons to the whole network [4]. Using the proposed model we reveal dynamical principles of bursting to superbursting mode switch driven by modification of key model parameters. Our results indicate that among the most crucial factors underlying superbursting phenomenon are the duration of excitatory postsynaptic currents, the balance between excitation and inhibition and short-term plasticity kinetics. The main findings are in line with experimental observations after digestion of the extracellular matrix.

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DEVELOPMENT OF NEW FLUORESCENT VOLTAGE SENSOR PROTEINS

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Abstract. We are developing fluorescent voltage sensor proteins that can be useful for optical recording of membrane potential in excitable cells. We created a series of FRET (fluorescence resonance energy transfer) based sensors, containing the voltage-sensing domain of CiVSP (*Ciona intestinalis* voltage sensitive phosphatase) with two fluorescent proteins (FP) inserted into different regions of the back bone. Insertion of FRET donor and acceptor at different locations affected plasma membrane expression, signal intensity, and response time. By examining FRET signals responding to changes of membrane potential in HEK293 cells, we identified novel probes with relatively large signals and fast kinetics.

Fluorescent voltage sensor proteins have excellent potential as a tool for imaging membrane excitability in live neurons. Unlike the first generation of sensor proteins utilizing voltage-gated ion channels, probes based on CiVSP can detect changes of membrane potential as a monomer. This improved the structural stability and membrane targeting of sensor proteins (Dimitrov et al., 2007). However, CiVSP based voltage sensors still suffer from slow kinetics.

In order to improve the performance of CiVSP based voltage sensors, we engineered a library of constructs with UKG (green-emitting fluorescent protein Umi-Kinoko) and mKO (monomeric orange-emitting fluorescent protein Kusabira orange) as FRET donor and acceptor (Tsutsui et al., 2008). By moving the insertion sites of 2 FPs around the voltage sensing transmembrane domain of the CiVSP backbone, we tried to systemically alter the interactions between the FRET pair and to improve signal intensity and kinetics of FRET response to changes of membrane potential. We evaluated voltage sensitive FRET signals in transiently transfected HEK293 cells at 33 °C and identified amino acid residues of CiVSP at which 2 FPs are inserted to generate good FRET. We identified novel voltage sensor proteins exhibiting large signal and fast kinetics, as an example shown in Fig. 2.

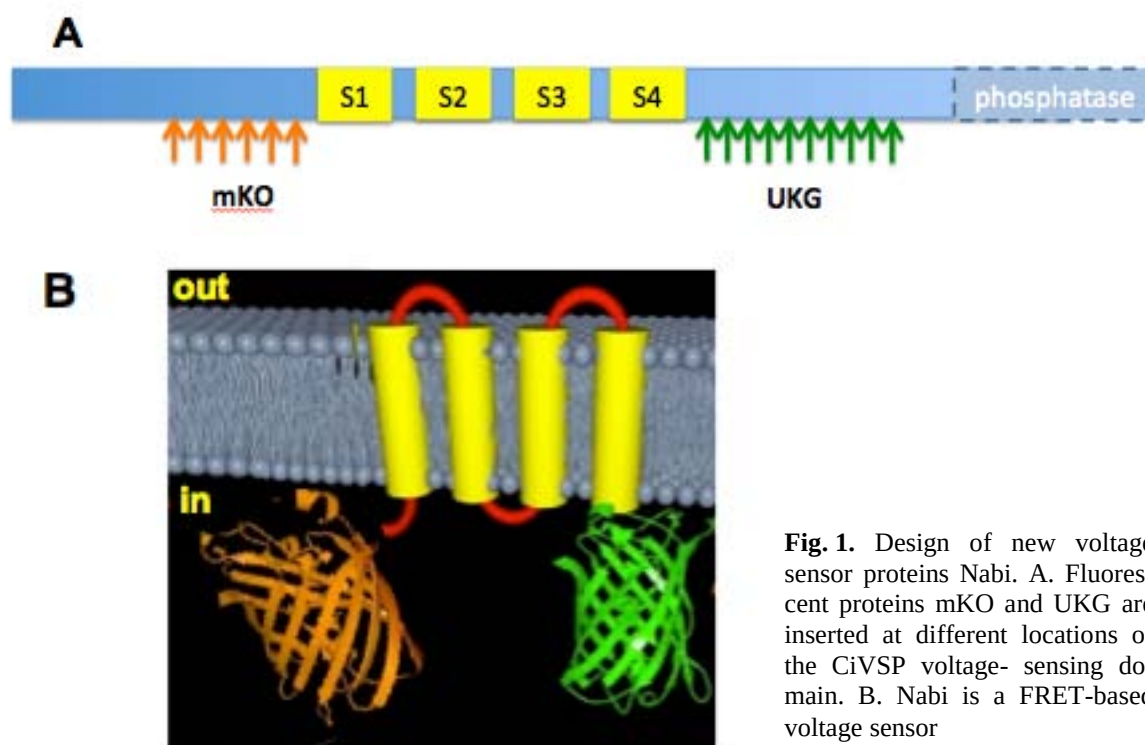


Fig. 1. Design of new voltage sensor proteins Nabi. A. Fluorescent proteins mKO and UKG are inserted at different locations of the CiVSP voltage-sensing domain. B. Nabi is a FRET-based voltage sensor

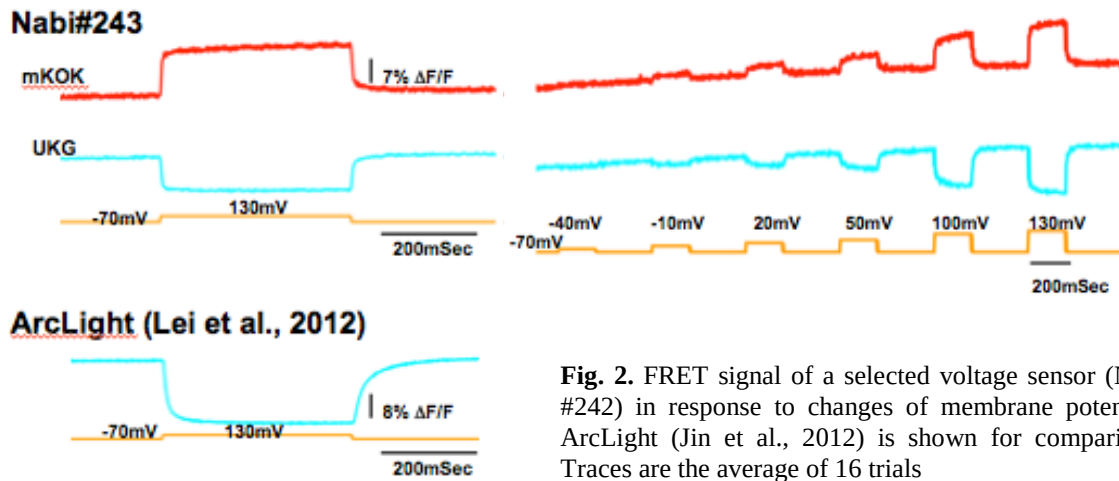


Fig. 2. FRET signal of a selected voltage sensor (Nabi #242) in response to changes of membrane potential. ArcLight (Jin et al., 2012) is shown for comparison. Traces are the average of 16 trials

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Invited

EXPLICIT REDUCED-ORDER INTEGRAL FORMULATIONS OF STATE AND PARAMETER ESTIMATION PROBLEMS FOR A CLASS OF NONLINEAR SYSTEMS WITH APPLICATIONS TO MODELLING OF ACTION POTENTIALS

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Abstract. We consider the problem of inferring unknown state and parameter values for systems of ordinary differential equations from measured data. For this problem, we discuss a solution based on re-formulation of the original problem as that of matching explicitly computable definite integrals with known kernels to data. This enables us to employ parallel computational streams and, consequently, increase the overall speed of calculations. If the measurement data is periodic the approach reduces effective dimension of the unknown parameters vector. Performance and practical implications of the method are illustrated with an example of fitting parameters of a conductance-based point model to data.

Consider a system governed by nonlinear ordinary differential equations

$$\dot{x} = f(x, t, p), \quad x(t_0) = x_0, \quad x_0 \in R^n, \quad (1)$$

where $f: R^n \times R \times R^k \rightarrow R^n$ is continuous and locally Lipschitz with respect to the variable x function, and p is the vector of unknown parameters. Let $[t_0, t_0 + T]$ be an interval on which the solution $x(\cdot, t_0, x_0, p)$ of (1) is defined. Let us further suppose that the system's state, $x(t, t_0, x_0, p)$, is not accessible for direct observation at any $[t_0, t_0 + T]$. One can, however, observe the values

$$h(t, x(t, t_0, x_0, p)), \quad h: R \times R^n \rightarrow R$$

for every $t \in [t_0, t_0 + T]$. The problem is to find $p' \in R^k, x'_0 \in R^n$ such that

$$h(t, x(t, t_0, x_0, p)) = h(t, x(t, t_0, x'_0, p')) \quad \forall t \in [t_0, t_0 + T]. \quad (2)$$

This is a standard inverse problem, and many methods for finding solutions to this problem have been developed to date (sensitivity functions [9], splines [3], interval analysis [6], adaptive observers [2, 4, 5, 8, 11, 12], and particle filters and Bayesian inference methods [1]). Despite these methods are based on different mathematical frameworks, they share a common feature: one is generally required to repeatedly find numerical solutions of nonlinear ordinary differential equations (ODEs) over given intervals of time (solve the direct problem).

Notwithstanding the plausibility of numerical integration of systems of ODEs in algorithms for state and parameter estimation, this operation is an inherently sequential process. This constrains computational scalability of the problem, and as a result imposes limitations on the time required to derive a solution. In order to overcome this limitation we propose to cast the inverse problem above in an alternative, integral form. In particular, instead of finding numerical solutions of the initial value problem (1) and matching the results to observed data, e.g. as (2) we search for a representation of the problem as

$$\begin{aligned} y(t) - F(p, x_0, t) &= 0 \quad \forall t \in [t_0, t_0 + T], \\ F(p, x_0, t) &= \int_{t_0}^t g(t, \tau, p, x_0) d\tau, \end{aligned} \quad (3)$$

where $g: R \times R \times R^k \times R^n \rightarrow R^d$ and $y: R \rightarrow R^d$ are functions that are explicitly computable from measurement data. Furthermore, we additionally require that if p', x'_0 is a solution of (3) then it is also a solution of (2) and vice-versa.

In the talk we specify a class of systems for which such representation is possible. This class of systems is not as general as (1) but is relevant enough in modelling applications. We will formally define this class of systems and present general technical assumptions. This is followed by presentation of main theoretical results. The results are based on the periodicity assumption we impose on the data and also on known facts from the theory of adaptive observers [7, 8]. The approach is illustrated with an example for state and parameter estimation of the Morris-Lecar model [10].

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QUANTITATIVE REAL-TIME NEUROIMAGING OF MULTIPLE FUNCTIONS BY LASER SCANNING MICROSCOPY

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Abstract. Fluorescent microscopy image brightness allows determining ion concentration with the help of single-wavelength specific indicator, whose fluorescence intensity changes at binding with ions inside cells. It permits to use only one monochromatic channel of laser scanning microscope for quantitative diagnostics of some function. It gives opportunity of network activity monitoring with multimodal description: to investigate concentration of different ions, potential distribution and morphology at the same time during temporal evolution.

Many cell functions are directly associated with concentration of one or another ion. Ion visualization is possible with the help of specific fluorescent indicator [1, 2]. Laser scanning microscopy as applied to biophotonics allows realize high resolution morphological and functional bioimaging.

Calcium ion concentration ($[Ca^{2+}]_i$) dramatically influence on brain cells vital activity. A critical evaluation of the role of calcium as an intracellular messenger requires quantitative measurements of $[Ca^{2+}]_i$, and comparisons of varied stimuli and cell responses. So at brain cells functional activity investigation reliable information of calcium ion concentration dynamics has great importance. This work is devoted to the problem of data reliability obtaining in experiments on rat and mice hippocampal cells with the help of laser scanning fluorescence microscopy for neuron-glia network signaling processes. We use Zeiss LSM510 NLO Duoscan confocal and two-photon microscope system for registration and preprocessing of fluorescence signals from hippocampal slices or cell cultures dyed by specific calcium indicator.

The most popular approach to measuring of intracellular free calcium ion concentration is ratiometric imaging using the UV excited calcium indicators offered by Grynkiewicz et. al. [1]. With this method, calcium ion concentration is calculated from a ratio of fluorescences at two excitation wavelengths. In our case $[Ca^{2+}]_i$ determinates with the help of single-wavelength specific calcium indicator Oregon Green BAPTA1 AM, which fluorescence intensity in green color wavelength band changes at binding with calcium ions inside neuronal and glial cells. For discerning of neurons and glia Sulforhodamin 101 as astrocytic marker is used. It penetrates into astrocytes and its red fluorescence together with green fluorescence of Oregon Green give composite yellow color of calcium-active glia along with green live neurons. Filtering Oregon Green fluorescence from everything else and measuring its intensity in principle it is possible to calculate calcium ion concentration but the known relationships between fluorescence and calcium ion concentration [1, 2] is difficult to use in practice because of necessity to know fluorescence intensities at maximum and minimum $[Ca^{2+}]_i$ for every concrete experimental situation.

We suggest $[Ca^{2+}]_i$ quantification method based on high affinity single-wavelength calcium indicator fluorescence intensity in situ measurement subject to dye loading protocol and fluorophore excitation conditions without calcium saturation and the reduction of resting $[Ca^{2+}]_i$ in every experiment *in vitro*. Fluorescence intensity F is determined by the product of the dye concentration n , excitation intensity I_0 , molar extinction coefficient α , fluorescence quantum yield Q_F , quantum yield of the photo-detector Q_D , and Φ – number of photons acquired by the optics :

$$F = \Phi Q_D Q_F I_0 n .$$

Since α and Q_F of free and Ca^{2+} -bounded dye molecules is different,

$$F = S_f n_f + S_b n_b ,$$

where n_f и n_b is concentration of free and Ca^{2+} -bounded dye, and

$$S = \Phi Q_D Q_F I_0 -$$

fluorescence intensity of dye concentration unit.

Using dissociation constant conception:

$$K_d = \frac{n_f [Ca^{2+}]_i}{n_b},$$

$$[Ca^{2+}]_i = K_d \frac{n_b(S_b - S_f)}{n_f(S_b - S_f)}.$$

And subject to total dye concentration $n_\Sigma = n_b + n_f$, finally:

$$[Ca^{2+}]_i = K_d \frac{F - S_f n_\Sigma}{S_b n_\Sigma - F}.$$

Thus we can determine calcium ion concentration from parameters of using dye (dissociation constant, also extinction coefficient and fluorescence quantum yield), instrument function of microscopy equipment, total amount of loaded dye that is the result of fill up protocol and measuring fluorescence intensity. It allows to calculate $[Ca^{2+}]_i$ in the course of experiment even in real time regime.

In the case of necessity to determine total calcium concentration, secreted at some metabolic process, it can be made by summation of free and bounded calcium:

$$[Ca^{2+}]_{total} = K_d \frac{F - S_f n_\Sigma}{S_b n_\Sigma - F} + \frac{F - S_f n_\Sigma}{S_b - S_f}.$$

We consider the problem of the calcium activity in neuronal tissue in the light of its mechanisms. Quantification of calcium ion concentration with the help of fluorescence analysis allows solving question of one or another of them domination at different functional states. Calcium signal transmission can come through voltage-dependent Ca-channels or ligand-gated calcium channels. Adding of blocker of postsynaptic receptors and/or by neurotransmitter application we can estimate the contribution of different types of calcium receptors in spontaneous neuronal activity in hippocampus acute slices.

Parameters of kinetic fluorescence for the adequate interpretation of the neuronal activity results in long-term recording signals were optimized. Relationship between fluorescent microscopy image brightness and operation factors of the indicator, excitation, optic and electronic devices allow determine ion concentration starting from measuring fluorescence intensity, loading protocol, optics and electronic units hardware function. In that way ion concentration is determined with the help of single-wavelength specific indicator, which fluorescence intensity changes at binding with ions inside neuronal and glial cells. In contrast to dual-wavelength quantification method it permits to use every channel of laser scanning microscope for diagnostics of individual function or structural measurements enlarging information density of real-time experiment. Unlike existing single-wavelength dye quantification by calcium saturation and the reduction of resting $[Ca^{2+}]_i$ in every experiment *in vitro*, suggested technique allows measuring ion concentration in all cells and saves cell culture in living state, hence it gives opportunity of network activity monitoring with quantitative multimodal description. Using it for neuron-glial network signaling investigation it is possible study more than one ion concentration cell distribution and its temporal evolution simultaneously.

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REGULATED EXOCYTOSIS: FUSION PORE INTERMEDIATES OF PEPTIDERGIC VESICLES

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Abstract. Exocytosis is a multistage process involving a merger between the vesicle and the plasma membranes, leading to the formation of a fusion pore, a channel, through which secretions are released from the vesicle to the cell exterior. A stimulus may influence the pore by either dilating it completely (full-fusion exocytosis) or mediating a reversible closure (transient exocytosis). In neurons, these transitions are short-lived and not accessible for experimentation. However, in some neuroendocrine cells, initial fusion pores may reopen several hundred times, indicating their stability. Moreover, these pores are too narrow to pass luminal molecules to the extracellular space, but their diameter can dilate upon stimulation. To explain the stability of the initial narrow fusion pores, anisotropic membrane constituents with non-axisymmetrical shape were proposed to accumulate in the fusion pore membrane. Although the nature of these is unclear, they may consist of lipids and proteins, including SNAREs, which may facilitate and regulate the pre- and post-fusional stages of exocytosis.

Exocytotic fusion pore intermediates

Neurotransmitters and hormones are stored in membrane-bound cytoplasmic secretory vesicles and are released into the extracellular space after fusion of the vesicle membrane with the plasma membrane. Membrane merger is considered energetically unfavourable, an event within the multistage process of regulated exocytosis. It is triggered typically by an increase in intracellular free Ca^{2+} , which is required for the merger between the vesicle and the plasma membranes, leading to the formation of the fusion pore, an aqueous connection between the vesicle lumen and the extracellular space. Since the distance between these two compartments is a few nanometers, the release of vesicle content may reach a timescale of micro- to milliseconds. Fusion pore formation is thought to be associated with conformational change in the soluble *N*-ethylmaleimide-sensitive factor activating protein receptor (SNARE) complex that is formed between the vesicle and the plasma membranes [1, 2]. Exocytotic fusion pore may exist as a pre-formed dynamic structure before the arrival of the stimulus [3, 4]. Initially the pore is narrow and prevents the discharge of vesicle content into the extracellular medium (termed unproductive exocytosis) [5]. However, this fusion pore intermediate may widen on stimulation, initiating rapid transmitter release [3, 4, 6]. Furthermore, the fusion pore diameter can fluctuate for several minutes before it irreversibly widens or closes. Such fluctuations may limit the discharge of vesicle content and therefore control transmitter release at the post-fusion stage of exocytosis [7]. Thus, although fusion pores were initially thought to be energetically unstable and short-lived structures, increasing evidence exists that fusion pores are subject to stabilization [8] and post-fusional regulation [3, 9].

Vesicles that enter into the process of regulated exocytosis consist of small synaptic vesicles (SSVs) and large dense-core vesicles (LDCVs), generated from the Golgi and are brought into close proximity to the plasma membrane before entering regulated exocytosis. In neurons, specialized protein-rich sites of release, termed active zones, exist on the plasma membrane and provide a molecular scaffold for docking of SSVs to the plasma membrane before fusion. However, neurotransmitters can also be released from SSVs in regions outside the active zone in a process termed ectopic release, which is common for LDCVs. In ectopic release, docked LDCVs are randomly distributed at the plasma membrane. But there are some exceptions. Recent results on pituitary lactotrophs and melanotrophs have revealed that the membrane composition at the sites of LDCV docking differs from the sites devoid of vesicles [10]. After docking, secretory vesicles are primed to respond to a Ca^{2+} -trigger, which leads to the release of cargo from the primed vesicle. At this stage, it is thought that a fusion pore is formed that enables diffusion of vesicle cargo from the lumen into extracellular space. Since active zones are rich in voltage-gated Ca^{2+} channels [11] SSVs that are primed in active zones respond to the increase in $[\text{Ca}^{2+}]_i$ faster than LDCVs and those SSVs that are randomly distributed at the plasma membrane, although the plasma membrane outside the active zones is not completely devoid of Ca^{2+} channels. Following the fusion, membrane components of SSVs and LDCVs are recycled in a clathrin- or dynamin-dependent mechanism [12]. SSVs are recycled locally within presynaptic bou-

tons. They are refilled with low molecular weight neurotransmitters by transporter-mediated uptake of neurotransmitters from the cytosol, driven by a pH gradient¹. On the other hand, LDCVs are formed *de novo*. Only vesicle membrane components of LDCVs are retrieved and recycled back to Golgi. LDCVs contain cargo proteins and peptides, which are synthesized in the endoplasmic reticulum, and while being transported through *trans*-Golgi networks they mature.

Properties of exocytotic fusion pores have been studied extensively in the past few decades using biophysical measurements, including high-resolution optical techniques such as fluorescence imaging [3, 13-16], amperometric recording [17] and electrophysiological techniques, including membrane capacitance measurements [18, 19]. The latter approach allows the monitoring of membrane area fluctuations due to exo- and endocytosis at the level of a single vesicle. Moreover, several fusion pore stages can be detected with this technique and one can study how proteins, lipids and cAMP-mediated mechanisms affect these events [6, 20, 21]. To determine the vesicle diameter we have used STED super-resolution microscopy [6].

By using secretory model cells from the pituitary, we have been able to demonstrate that after formation, fusion pore diameters may either expand irreversibly (i.e. full- fusion exocytosis) [22] or may first open but then rapidly close again (i.e. transient exocytosis) [23]. The repetitive reopenings of the fusion pore indicate that this intermediate is stable [6, 21]. Transient and full-fusion exocytosis may coexist in the same cell and have been reported for both SSVs [24] and LDCVs [3, 18, 24], although with the techniques currently available it is far more difficult to distinguish different modes of exocytosis for SSVs due to their small size (diameter of SSVs ranges between 40 and 80 nm) and other technical limitations than for LDCVs, which are 2–10 times larger than SSVs [24].

It is proposed that fusion pore intermediates that we have been able to determine in peptidergic LDCVs are also present in SSVs. The pre-existing narrow fusion pore (release unproductive) may contribute to the minimization of the stimulus-secretion delay.

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LUMINESCENT NANOMATERIALS FOR MOLECULAR-SPECIFIC BIOMEDICAL IMAGING

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Abstract. Luminescent nanoparticles have emerged as promising optical bioprobes due to their excellent photostability, tunable, narrow luminescence spectra, controllable size, and large surface area for anchoring targeting biomolecules. Some types of nanoparticles provide enhanced detection contrast due to long luminescent lifetime, and/or “anti-Stokes” spectral shift. Optical, physical and chemical properties of biocompatible luminescent nanocomplexes are discussed on the examples of quantum dots (QD), luminescent nanodiamonds (LND), nanorubies and upconversion nanoparticles (UCNP). We report on a whole bottom-up bio-nano-optics approach for optical biological imaging capturing luminescent nanoparticle design, surface activation and bioconjugation and the resultant bioconjugate module deployment in specific internalisation in cells and live biological tissue.

Introduction

Optical imaging assisted by molecular-specific labelling with luminescent nanoparticles (NPs) represents one of the most powerful manifestations of the nanotechnology impact in the life sciences. This is a direct, minimally invasive approach to investigate cellular morphology and processes in living cells and/or tissues, in their comprehensive biological context. The detection sensitivity can be pushed to the single molecule imaging unobscured by the ensemble averaging, which has become a very useful and common imaging modality suitable for complex biological systems. The fluorophore performance, however, is frequently compromised by undesirable photophysical properties, such as photobleaching that hinder its photostability. The other shortfalls of the fluorescence dyes include potential cytotoxicity, high susceptibility to aggressive chemical and biological environment and limited number of anchoring terminals to attach functional modules, such as drug molecules, biomolecules and nanoparticles.

Nanotechnology has provided a powerful impetus to a new generation of molecular probes based on luminescent nanoparticles that are capable of addressing major shortfalls of the existing molecular probes. In particular, a variety of nanoparticles, including luminescent nanodiamonds (LND), nanorubies and upconversion NPs (UCNPs) are eminently photostable whose emission is unfading and continuous. Besides, reduced cytotoxicity of these nanomaterials has been recently reported. The upconversion NPs and nanorubies offer an improved signal-to-noise ratio in the time-gated imaging modality and high-contrast detection employing time-gated schemes that have been reported using lanthanide-based UCNPs [1]. NP surface is amenable to modification to enable grafting a variety of surface moieties suitable for applications, such as molecular targeted drug delivery. These bioconjugated modules, developed by us, e.g. nanodiamond-antibody, (quantum dot)-(peptide somatostatin) or (upconversion nanoparticle)-(mini-antibody) can gain admission into the cells by initiating cell-specific, cell-recognised communication protocol. In this paper, we aim to demonstrate a whole bottom-up bio-nano-optics approach for optical biological imaging capturing luminescent nanoparticle design, surface activation and bioconjugation and the resultant bio-conjugate module deployment in specific internalisation in cells and live biological tissue.

Results

We will review several types of novel luminescent nanoparticles, including luminescent nanodiamonds, nanorubies, and upconversion nanoparticles, in the context of optical cellular/tissue imaging benchmarked against established semiconductor quantum dots (QDs) nanotechnology. The rationale behind the choices of these NPs is

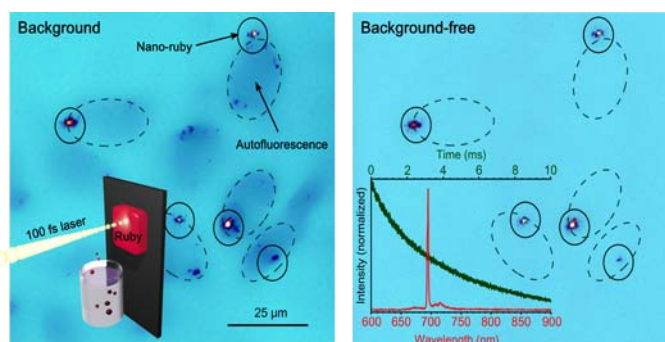


Fig. 1. Scanning confocal microscopy background-free imaging of nanoruby in CHO cells

the diversity of their luminescence properties that represent important classes of nano-emitters fitting into key biomedical imaging application niches, such as high-contrast high-sensitivity imaging on the crowded background of the scattering biomatter structure and cells/tissue autofluorescence. Such imaging performance was demonstrated by using an example of ruby nanoparticles produced by the femto-second laser ablation (Fig. 1, *left panel*, inset) that exhibited very narrow spectral width of a few nanometres at the emission centre wavelength of 694 nm falling into the biological matter transparency window, and exceptionally long emission lifetime (3 ms), which was c.a. million-fold of that of intrinsic fluorophores (autofluorescence) of almost all biological systems, e.g. cells shown in Fig. 1 [1]. This enabled employment of time-gated detection scheme, where the excitation back-scattered light and autofluorescence signals were completely suppressed leading to the ultrahigh-sensitivity background-free detection, i.e. at the level of a single nanoparticles mean-sized 12 nm, as shown in Fig. 1, *right panel*.

We address a promising NP biocomplex self-assembly approach based on a high-affinity molecular linkers, such as avidin:biotin and barnase:barstar, which provides crucial simplicity and flexibility for designing and assembling nanoparticulate biocomplexes of variable complexity using preformed sub-

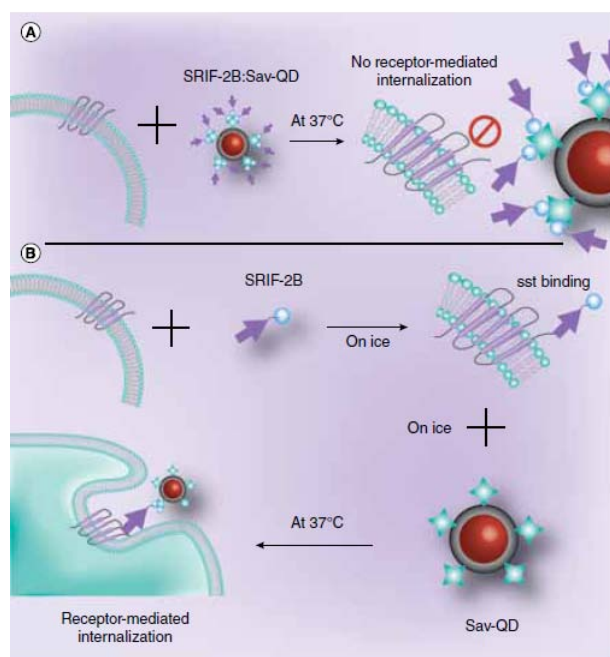


Fig. 2. Schematic diagram explaining in situ two-step conjugation strategy of SRIF (somatostatin) peptide and quantum dot (QD) for specific targeting of somatostatin receptors (sst)

units each of which is equipped with one of the two linkers [2]. The external subunit contains a targeting vector, which can be a mini-antibody raised against the epithelial growth factor receptors HER2/neu for specific delivery of upconversion nanoparticles in adenocarcinoma cells [3]; or a peptide enkephalin, which, when grafted onto a nanoparticle complex, is specifically internalised via opioid-receptor mediated endocytosis in neuron-like cells. We also report, for the first time, on the development of a quantum dot (QD)-based luminescent somatostatin (SRIF) probe that enables specific targeting of somatostatin receptors. Receptor-mediated endocytosis of SRIF was imaged using the developed probe that contained a luminescent nanoparticle (QD) contrast agent. A biotinylated analogue of SST (SST-2B) was conjugated to a streptavidin-coated QD (Sav-QD), forming an SST-2B:Sav-QD complex. An *in situ* two-step conjugation strategy was introduced to overcome the Sav-induced inhibitory effect, an approach that demonstrated to be successful for specific targeting of the somatostatin receptors [4], with the result presented in Fig. 2. Their

deployment for the targeted delivery into cells that express somatostatin receptors, for example, in brain regions responsible for the regulation of blood pressure will be valuable.

Acknowledgements

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MATHEMATICAL MODEL OF INTERNEURON FIRING DRIVEN BY EXCITATORY AND INHIBITORY INPUTS COORDINATED BY ASTROCYTE

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The effects of astrocyte influence on synaptic transmission were intensely studied in recent years [1]. It was found, in particular, that the release of gliatransmitters, which can be neuronal activity-dependent, can change the efficacy of synaptic transmission by acting on pre- and/or postsynaptic parts of the synapse [2]. For example, such changes may provide noticeable amplitude and frequency modulation of neuronal responses [3]. Astrocytes may influence the synaptic transmission in both excitatory glutamatergic synapses and inhibitory GABAergic synapses [4,5]. Many models of synaptic transmission modulated by astrocytes were proposed [2,3,6,7]. Most of them demonstrated how the astrocyte may stimulate facilitation and depression of excitatory transmission.

In this work we considered an interneuron that typically received both excitatory and inhibitory inputs accompanied by astrocyte. Based on experimental data we constructed a phenomenological model of heterosynaptic regulation of the interneuron firing by the astrocyte (fig. 1A). The model is composed of the interneuron with glutamatergic and GABAergic synapses and astrocyte influencing both inputs. The interneuron responses were described by Hodgkin-Huxley like nonlinear differential equations driven by input synaptic currents [8]. Next, the dynamics of the neurotransmitters and the gliatransmitters were defined by a set of linear differential equations with different rates for different kind of molecules. The postsynaptic dynamics was modeled by a gamma-function distribution of postsynaptic current amplitudes that fit qualitatively the experimental observations. We assumed that the excitatory synaptic input may be modulated by glutamate released by the astrocyte in two distinct ways. In the first case, the glutamate bound to presynaptic NMDA receptors may increase the release probability. In the second case, the glutamate may depress the release when bound to metabotropic glutamate receptors. The postsynaptic part can be influenced by D-serine that activates postsynaptic NMDA receptors and increases EPSCs amplitudes. The inhibitory input was modulated by the glutamate released by the astrocyte, which activated axonal kainate receptors leading to the increase in the frequency of spontaneous inhibitory postsynaptic currents (IPSCs)[5].

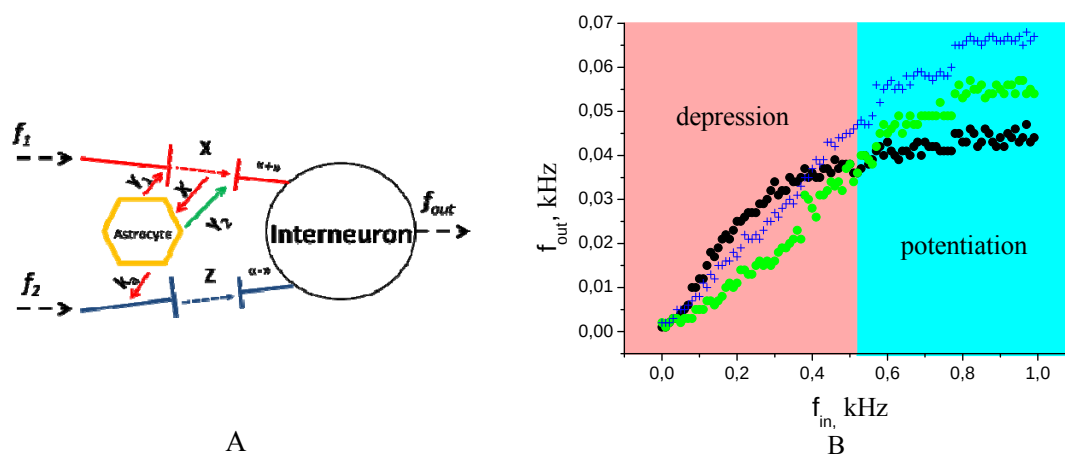


Figure 1 – A. Model of the astrocyte mediated coordination of excitatory and inhibitory inputs to interneuron, where X is the concentration of neurotransmitter (glutamate), Z is the concentration of GABA, Y_1 and Y_2 are the concentration of glutamate and D-serine released by the astrocyte, respectively, k_a is the parameter dependent on Y_1 and defining frequency scaling in the inhibitory synapse. B. Dependence of the

interneuron response frequency, f_{out} , on the frequency of input signals, f_{in} . Black, blue and green curves were calculated for the inactive astrocyte, astrocyte modulated only the excitatory input and astrocyte influenced to both inputs, respectively.

We assumed that the interneuron is an element of a spiking network and, hence, in spontaneous dynamics the statistics of presynaptic activations can be described by Poisson spike train with average frequency f_{in} . Then, the average input frequencies for the two inputs can be expressed by $f_1 = f_0^1 + f_{in}$ for the excitatory input and $f_2 = k_a f_0^2 + k_s f_{in}$ for the inhibitory one, respectively, where $f_0^{1,2}$ are the frequencies of spontaneous neurotransmitter release, $k_a = k_a(Y_1)$ is the coefficient of frequency scaling by the astrocyte glutamate and k_s is the correlation coefficient between the two inputs.

We analyzed the dependence of the output spiking rate of the interneuron on the input frequency f_{in} (figure 1B). Interestingly, the effect of the astrocyte regulation was frequency specific. Particularly, for relatively low frequencies the astrocyte depressed the synaptic transmission (blue and green dots in fig.1B). For the higher frequencies the response was increased due to the increase of the EPSCs amplitudes (blue dots) and further attenuated by the effect of the inhibitory input facilitated by the astrocyte (green dots). Note that the facilitation of the inhibitory input can drastically decrease the response as illustrated in fig.2A. The astrocyte locked the synaptic transmission for the lower frequency working as a “high pass filter” (fig.2B).

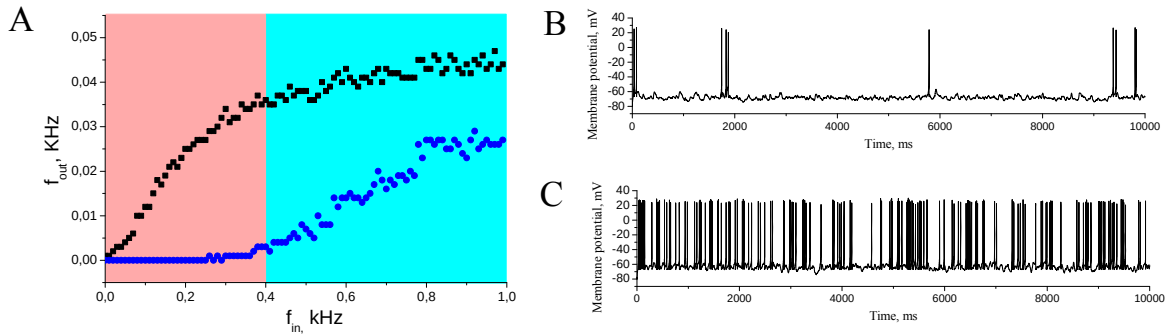


Figure 2 – A. Dependence of the interneuron response frequency, f_{out} , on the frequency of input signals, f_{in} . The output spike trains for red area (B) and blue area (C) are presented on the right.

In summary, the model predicted that the astrocyte can effectively regulate the interneuron firing by coordinating the excitatory and inhibitory synaptic inputs. In particular, it permits network activity-dependent increase or decrease of the output firing rate in certain frequency ranges.

Acknowledgements

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IMPACT OF LOW-INTENSITY LASER RADIATION WITH WAVELENGTHS OF 405 AND 475 nm ON SPERMATOGENESIS OF MALE RATS

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Abstract. The goal of the work is to study the impact of low-intensity laser radiation with wavelengths of 405 and 475 nm on spermatogenesis of male rats. To assess oxidative processes the method of induced chemiluminescence was used. Irradiation of testes by low-intensity laser radiation leads to primary intensification of free-radical and antioxidant activity of ejaculate and increases the number and motility of sperm cells. A week after discontinuation of exposure the measured parameters of free-radical activity are restored to the previous values; the spermatogenesis stimulating effect persists. The laser exposure wavelength of 405 nm is more effective for stimulation of spermatogenesis.

Reproductive system is one of the most sensitive and vulnerable in the body. Male infertility is responsible for 30-50% of infertile couples. The Socio-economic importance of fertility is the reason of high interest of modern andrology to the problem of male fertility decline and to finding new methods of stimulation of spermatogenesis. The positive impact of laser radiation on spermatogenesis and sperm in vitro was reported in the medical literature. It is known that the absorption of light energy by spermatozoa leads to participation of the photon energy in biochemical transformations. Exposure of sperm cells to red laser radiation in vitro resulted in increased activity of enzyme systems and, hence, in increased period of sperm motility. These data suggest that low-intensity laser radiation improves the functional state of spermatozoa due to direct local action.

The work aims at assessing the effect of low-intensity laser radiation at the wavelengths of 405 and 475 nm on spermatogenesis of laboratory animals and at studying the relationship between free radical processes and morpho-functional characteristics of sperm of albino rats under the action of low-intensity laser radiation.

Materials and methods. The experiments were performed on male outbred albino nonlinear rats aged 5-7 months. The material under study was ejaculate. The oxidative processes were assessed using the method of induced chemiluminescence. The ejaculates for research were obtained by intraperitoneal injection of oxytocin. We take into account separately cells with progressive, vibrational motility and stationary spermatozoa.

Results. Irradiation of testes of experimental animals by low-intensity laser radiation for 10 days leads to primary intensification of free-radical and antioxidant activity of the ejaculate. Compared with parameters of intact animals, free-radical and antioxidant activity of ejaculate after irradiation of testes by low-intensity laser radiation increased by 42% and 52%. Free-radical and antioxidant activity of ejaculate after irradiation of testes by visible light were also up to 35% and 69%. A week after discontinuation of exposure the measured parameters of peroxidation and antioxidant activity in ejaculate restored to the previous values. This primary activation of free-radical and antioxidant activity is likely to be a compensatory response.

The study of ejaculate is one of the most informative methods for the assessment of male reproductive system. The action of low-intensity laser radiation in vitro increases sperm motility by 80% due to the intensification of processes of cellular metabolism (Fig. 1).

The abundance of sperm in the ejaculate of intact animals throughout in vivo experiment was permanent and amounted to 3.30 ± 0.15 mln (Fig. 2). The number and motility of sperm cells in the ejaculate of the control group did not significantly differ from the parameters of intact rats. The number of sperm cells in the ejaculate after irradiation of testes by low-intensity laser radiation with wavelengths of 405 and 475 nm increased up to 5.30 ± 0.12 mln and 4.57 ± 0.18 mln, respectively. It was shown that irradiation of testes by low-intensity laser radiation stimulates sperm motility by 10%. The effect of stimulation of spermatogenesis persisted a week after discontinuation of exposure.

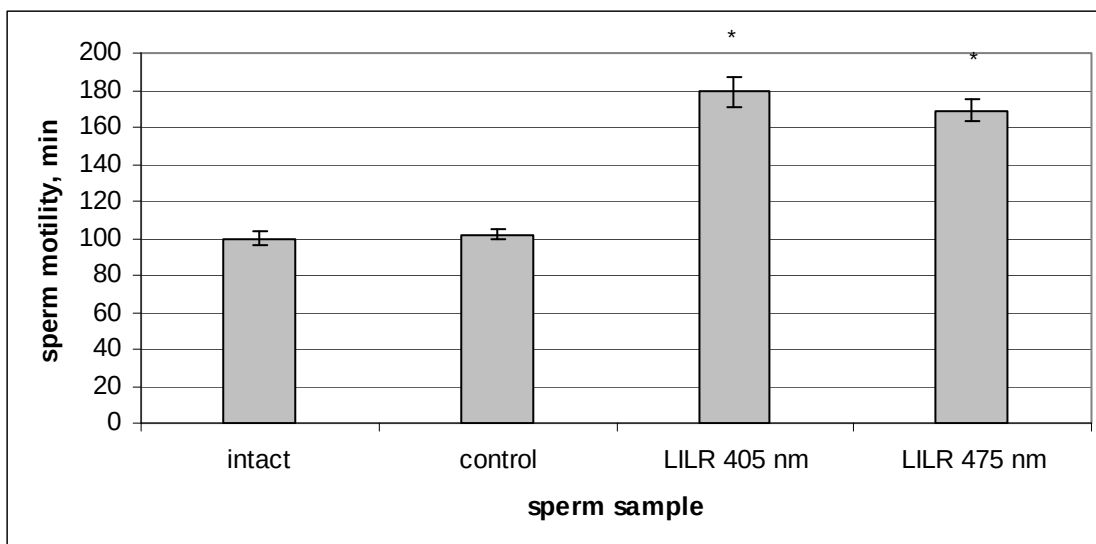


Fig. 1. Changing sperm motility under the action of low-intensity laser radiation (LILR) with wavelengths of 405 and 475 nm in vitro; * – statistically significant differences compared with the group of intact animals

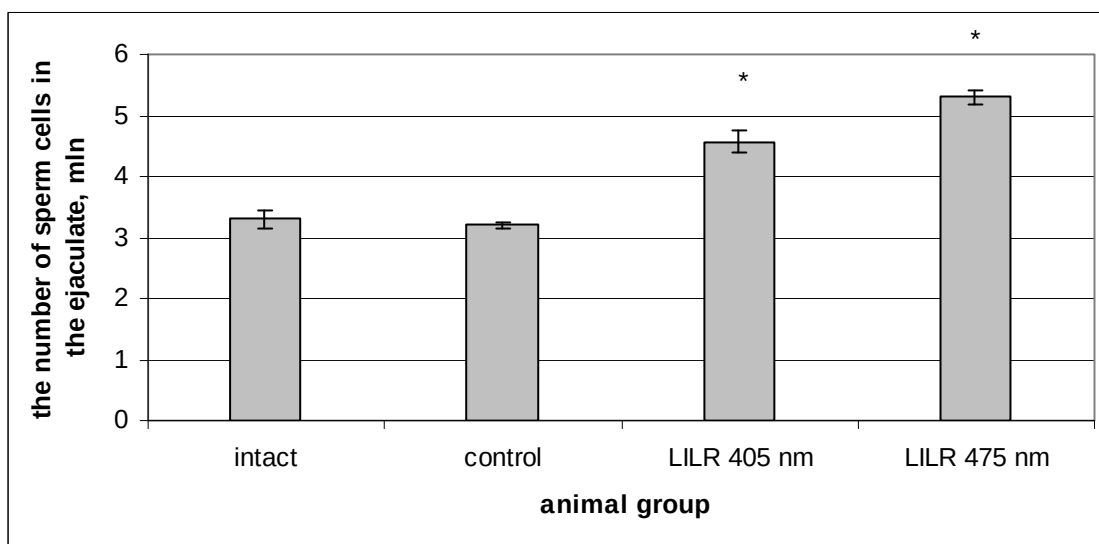


Fig. 2. Changing number of sperm cells in the ejaculate of rats under the action of low-intensity laser radiation (LILR) with wavelengths of 405 and 475 nm; * – statistically significant differences compared with the group of intact animals

To conclude, low-intensity laser radiation at the wavelength of 405 and 475 nm (course – 10 days, duration of exposure – 1 min, distance to the scrotum – 1 cm) leads to stimulation of spermatogenesis without causing an imbalance in pro-/antioxidant systems. The laser exposure wavelength of 405 nm is more effective for stimulation of spermatogenesis. A high correlation has been revealed between the quantity and motility of sperm and the parameters of free radical activity.

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LASER-INDUCED ALTERATION OF BIOMECHANICAL PROPERTIES AND SHAPE OF COSTAL CARTILAGE IN APPLICATION TO PECTUS EXAVATUM REPAIR

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Abstract. The development of a laser procedure for correction of deformities of pectus excavatum requires careful consideration of peculiarities the object under study. These peculiarities include biochemical composition, dimensions and preservation of surrounding tissues under laser heating *in vivo*. In this study we proved that porcine costal cartilage is a suitable model for studying human costal cartilage. It was shown that heating of cartilage with 1.68 μ m laser irradiation provides stress relaxation and allows rapid attainment of a stable steady-state geometry.

Laser cartilage reshaping is a novel technique designed to permanently alter the shape of cartilaginous structures without the use of scalpels, scoring, or morselization. Laser reshaping has also been used clinically in septoplasty operations and to perform otoplasty [1, 2]. The goal of this study is to develop the basis of a laser procedure for correction of deformities of pectus excavatum. We use porcine rib cartilage as a model unit.

Results

Chemical composition and thermal stability

Glycosaminoglycan (GAG) content was estimated using the colorimetric DMMB assay [3]. The collagen content was estimated from amino acid analysis of acid hydrolysates of tissue assuming that hydroxyproline makes up 13.3% of the collagen molecule weight. The molar ratio of hydroxyproline/hydroxylysine was used as the collagen type classifier. This value indicated that the costal cartilage main content was type II collagen [4].

Table 1. Chemical composition of the costal cartilage of various species

Samples	GAG, mg/g dry weight	Collagen, mg/g dry weight	Hyp/Hyl
Porcine CC	18.5 $\pm$ 2.2	32 $\pm$ 2.2	5.2 $\pm$ 0.2
Human PE CC	19.92 $\pm$ 4.45	31 $\pm$ 2.2	4.8 $\pm$ 0.3

Thermal stability was studied by differential scanning calorimetry (Netzsch DSC 204). It was found that collagen macromolecules in porcine CC and some fragments of PE human CC are stable up to 110°C. But partial collagen denaturation occurs at heating of PE human CC fragments in individual cases. The biochemical and thermal analyses of the samples did not show distinction of kind between porcine and human PE CC.

Laser parameters selection

Temperature of stress relaxation is known to be no less than 70°C. Then the maximal temperature of

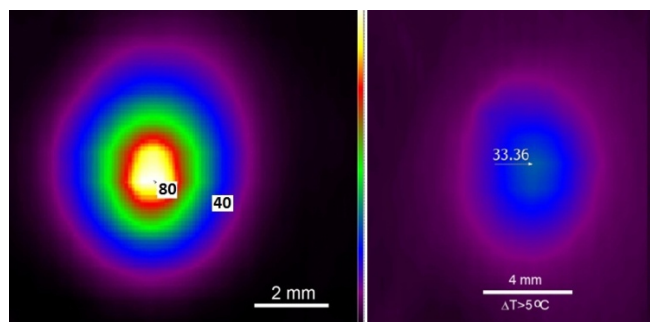


Fig. 1. Temperature field after 10 s, 3 W, 100ms/100ms laser treatment. cross-sectional view in the plane of 5 mm depth (left) and 3 mm depth (right)

the cartilage posterior surface cannot exceed 45°C. Then the laser procedure requires specific three-dimensional but not uniform heating of the tissue. For biological tissues the penetration depth of light at 1.68 μ m was assessed to be about 2 - 4 mm and a 1.68 μ m fiber laser radiation was applied to achieve specific temperature distribution. An infrared imaging system (IRTIS 200) was used to determine the temperature distribution in the tissue space.

The laser energy was delivered via a 600- μ m multimode silica fiber positioned per-

pendicular to the specimen and touched it. Laser power, pulse/interval duration and exposure time were varied. It was found that the optimal laser power is 2 - 3.5 W. The pulse/interval duration is from 300/100 ms to 50/50 ms. The exposure time does not exceed 25s. The examples of temperature fields at the 3 mm and 5 mm depth of costal cartilage are presented in the fig. 1.

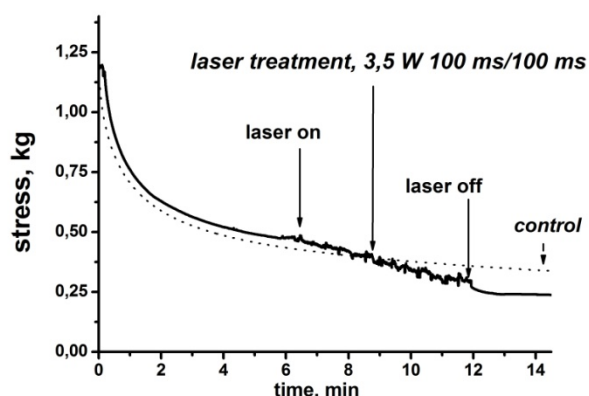


Fig. 2. Stress relaxation acceleration during laser treatment

The number of laser spots to get stress relaxation was obtained from the experiments with recording the instantaneous mechanical force response of the warping costal cartilage to laser influence. Large (5×5×40 mm) curved blocks were placed in a custom jig assembly designed to maintain the specimen in a curved semi-circular geometry. The specimen holder was mounted onto the table of a 1.5-kg mechanical testing machine. The laser spots were staggered along 2 parallel rows. Figure 2 demonstrates reaction force-time dependences of the response of costal cartilage. The example illustrates the changes of mechanical properties of costal cartilage during laser treatment.

Long-term effect of the new cartilage shape stability

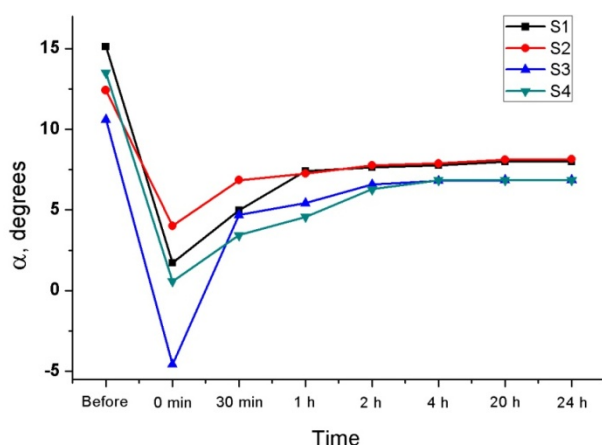


Fig. 3. Warping angle vs time for costal cartilage unit in the laser exposure group

A total of 27 porcine costal cartilage units (diameter 6-8 mm) were tested. Prior to laser treatment a cartilage bend angle was measured and then the specimen was bent with a jig composed of two metallic clamps. Next, the cartilage unit was irradiated for 10-15 s with a fiber laser (2.3-3.5 W, 10-12 spot). Then clamps were released and the unit was immersed in saline solution at ambient temperature. The warping angle was determined at 0 min, 30 min, 1 hour, 2, 3, 4, 20 hours, and 24 hours. The units underwent accelerated shape change within the first 30 minutes to reach a stable geometry (fig. 3).

Conclusion

Laser irradiation at 1.68 μm of costal cartilage provides an effective method for rapidly stabilizing acute shape change by accelerating the warping process.

Acknowledgements

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ADVANCES IN THERMO OPTICALLY POWERED SURGERY

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Abstract. Contact surgery with hot tip is dominating mechanism of diode laser surgery in near infrared range. Direct non-contact cutting with laser power alone requires high power level beyond capabilities of diode lasers available for dentistry. Temperature in contact surgery has been found to be a dominant factor in soft tissue cutting and coagulation. Real-time control of tip temperature allows for a significant increase in safety, minimizing collateral thermal damage, and improving speed and precision of cutting. Control of tip temperature is made possible by a tip initiation procedure and a newly developed Automatic Power Control (APC) technology. The Thermo-Optically Powered (TOP) tip has a tissue cutting and coagulation effect due to thermal conduction and incandescent thermal radiation with an effective coefficient of tissue absorption close to that of a CO₂ laser. TOP surgery clinical examples will also be presented.

ASPECTS OF DIODE PUMPED Er:YAG LASERS FOR MEDICAL APPLICATIONS

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Abstract. Actually diode pumped Er:YAG lasers become available, and might be an alternative to flash-lamp pumped Er:YAG lasers for medical applications. The main difference in laser output is a higher maximum pulse repetition rate (1 kHz compared to 100 Hz) on the expense of lower pulse energy (50 mJ vs. 1 J). The objective of our study was to investigate the achievable quality and quantity of tissue removal for a variety of tissue types. In sum, the experiments reveal that precise and deep cuts can be performed in soft and hard tissue, except enamel.

Introduction

Beginning 25 years ago, tissue ablation by flash-lamp pumped Er:YAG lasers has been investigated, and as a result a variety of clinical applications have been established, especially in dentistry and dermatology. These lasers emit high energy laser pulses of up to 1 J, with a pulse duration of typically a fraction of a ms. Typical pulse repetition rates are in the range of some Hz to some 10 Hz, resulting in an average power of several W. As an alternative, actually a novel diode pumped Er:YAG laser system (Pantec Engineering AG) has become available. It has the same advantageous wavelength of 2.94 μm (strong absorption by water), and a relative high mean power of up to 15 W. Compared to the established systems, the diode pumped laser has the advantage of higher laser efficiency, smaller size, and less maintenance. Regarding to the tissue effect, the main difference to the flashlamp pumped lasers is a potentially high pulse repetition rate of up to 1 kHz, realized on the expense of the pulse energy. The objective of our study was to investigate the achievable quality and quantity of tissue removal, and the variability of thermal side effects controlled by the repetition rate.

Materials and Methods

For reproducible experiments, an experimental set-up was realized with computer controlled movement of the samples and possibility to moisten them by a water spray (Fig. 1). In a second setup the samples were irradiated under water via an optical fiber ($\varnothing 200 \mu\text{m}$; length 20 mm).

The used diode pumped Er:YAG laser (DPM-15, Pantec Engineering) provides an adjustable mean laser power up to 15 W, a pulse repetition rate ranging from 200 Hz to 2 kHz, and a pulse duration of 10 μs to 200 μs . Because shorter pulse durations result in lower pulse energies, in most cases the maximum pulse duration of 200 μs was used.

In order to achieve a radiant exposure typical for flash lamp pumped Er:YAG laser ablation (some 10 J/cm^2) laser light was focused to a spot size of 200 μm or 220 μm , respectively, with a nearly top hat profile. For this lens combinations were designed by optical ray tracing simulations (Zemax) based on measured laser beam divergence.

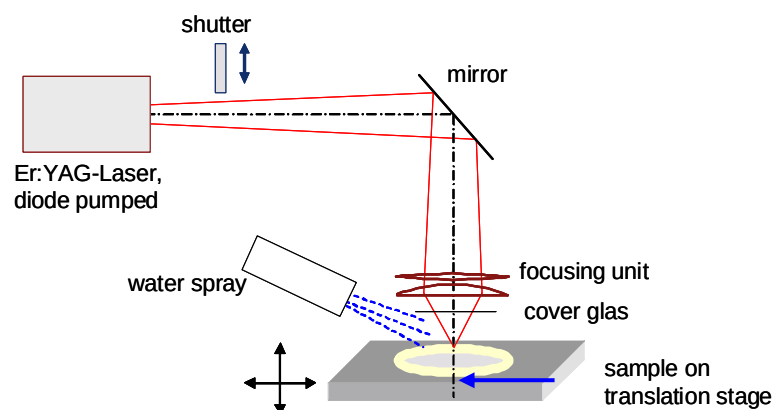


Fig. 1. Experimental setup. The sample is moved towards the water spray

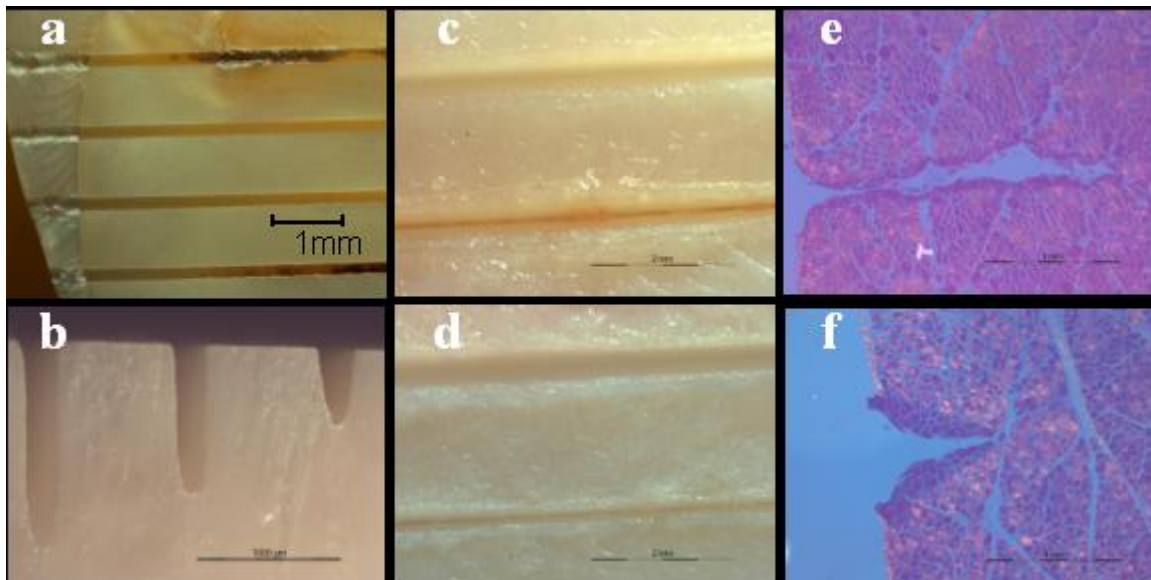


Fig. 2. Examples of experimental results (parameters given top down or left to right): a) tooth; with water spray; 1) 2.5 W / 100 Hz / 5 mm·s⁻¹, 2) same; 3) 3 W / 100 Hz / 5 mm⁻¹, 4) 5 W / 200 Hz / 10 mm⁻¹
b) bone, with water spray; 2.5 W / 100 Hz / 5 mm·s⁻¹; 5 / 3 / 1 passes (side view)
c, d) cartilage, without (c) and with (d) water spray; 1) 5 W / 200 Hz / 5 mm⁻¹, 2) 2.5 W / 100 Hz / 5 mm·s⁻¹
e, f) chicken breast, without water spray; e) 1.5 W / 50 Hz / 2 mm⁻¹, f) 1.5 W / 400 Hz / 2 mm⁻¹

Samples were mounted on a computer controlled translation stage for defined movement. When water spray was used for reducing thermal side effects, movement direction was thus that the moistened part of the sample was irradiated. After irradiation with various parameters the cuts were analyzed by light microscopy and laser scanning microscopy regarding to the ablation quality, the groove geometry, the ablation efficacy, and the thermal effects.

Investigations were performed on enamel and dentin (sections of human teeth, stored in 4% formaldehyde), bone, cartilage and skin from pigs, and chicken breast (all utilized freshly after buying from butcher).

Results

Representative images are given in Fig. 2. The observations and measured data can be summarized as follows:

Enamel: The cutting ablation quality observed with the tested parameters appears to be insufficient.

Dentin: Dentin can be ablated well, if sufficient water is supplied. The analysis of the grooves reveals an ablation efficiency of 0.124 mm³/J, which is comparable to that measured for flash lamp pumped Er:YAG lasers.

Bone: With appropriate water spray cutting quality is excellent. Too little moistening leads to carbonization, too much water reduces ablation depth. For optimal moistening, groove depth measured as a function of pulse energy results in a slope efficiency of 0.124 mm³/J, which is comparable to that of dentin. Multiple passes lead to a stalling increase in depth. Cutting under water is also possible.

Cartilage: Also for cartilage water spray is useful to improve cutting quality. A cutting depth exceeding 1 mm can be made with a single pass.

Skin, chicken breast: Smooth and deep (> 2 mm) cuts can be made with a single pass, water spray is not necessary. Cutting depth and coagulation zone are influenced strongly by the repetition rate (see Fig. 2, e, f), same power), speed of movement and power. Coagulation depth can be tuned from approximately 70 µm up to more than 400 µm.

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LASER PATTERNED MICROCOAGULATION FOR ORAL TISSUES TREATMENT

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Abstract. We initiated oral soft tissue regeneration using a minimally invasive microsurgical approach – Laser Patterned Microcoagulation (LPM). It is similar to the use of fractional photothermolysis that has been successfully applied in dermatology and ophthalmology. Regeneration of tissue structure is completed 28 days after laser treatment. The combined use of optical techniques (laser scanning microscopy based on second harmonic generation, cross-polarization optical coherence tomography and light polarization microscopy) allows assessing collagen state in the area of laser column at several levels of structural organization.

Periodontal soft tissue regeneration is one of the important objectives in periodontal therapy. In this study a minimally invasive microsurgical approach, Laser Patterned Microcoagulation (LPM), has been used to initiate gingival or oral mucosa tissue regeneration. The ultimate goal of the research is to create a new minimally invasive and cost effective treatment device and a procedure for gingival regeneration and regrowth. The study is designed to perform a feasibility assessment and histological observation of laser damage formation and regeneration process in the gingival and oral mucosa using an animal model. The LPM treatment concept is similar to the use of fractional photothermolysis [1], which has been successfully applied in dermatology [1, 2] and ophthalmology. Oral tissues are known to have even greater regeneration potential than skin [3] because of higher vascularization and faster metabolism. So they are supposed to experience fast scar-free regeneration.

Eighteen healthy rabbits underwent a single laser treatment in vivo in the gingiva and oral mucosa of the rabbit's maxilla at different time points. The rabbits were followed for up to 90 days. A diode laser operating at 980 nm wavelength and generating up to 20 W of average power was used. Each single column was created by a contact application of a tip 400 μm in diameter (Fig. 1) and irradiating the tissue through the tip with a single pulse with duration 80, 120 and 150 ms.

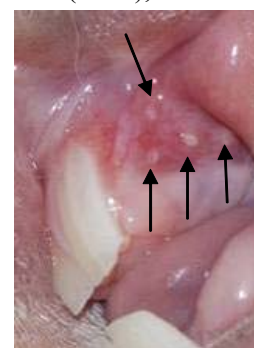


Fig. 1. View of laser columns on the rabbit's maxilla

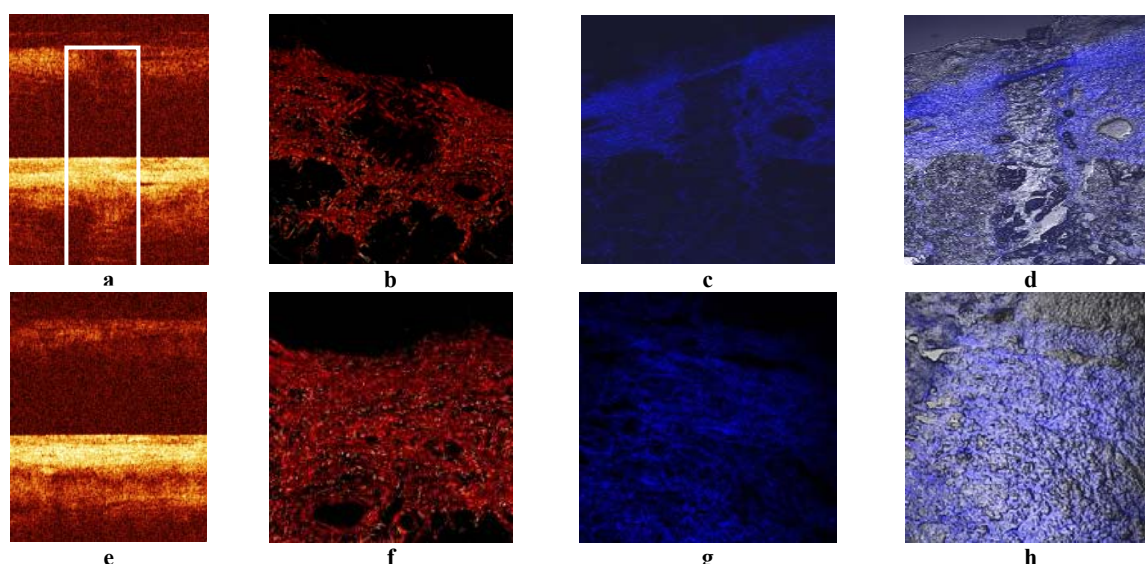


Fig. 2. Laser column healing: a - d – immediately after laser treatment; e - h – 28 days after laser treatment. CP OCT image in co-polarization (bottom) and in cross-polarization (top) (a, e), PM image with PSR staining (b, f), SHG image (c, d, g, h). SHG image size (c, d) is 0.95 x 0.98 mm. PM (b, f) and SHG image size (g, h) is 0.98x0.98 mm. CP OCT image size is 1.7 mm

Experimental study of the microdamage formation, healing and regeneration processes of oral soft tissues in animals on LPM using light, polarization and laser scanning microscopy based on second harmonic generation (SHG) and cross-polarization optical coherence tomography (CP OCT) showed that laser wound healing occurs in successive, overlapping stages: reactive inflammation, re-epithelization, reparation and regeneration. Regeneration of tissue structure is completed 28 days after laser treatment; no undesirable histological effects (dyskeratosis and epithelial spongiosis, subepithelial degenerative stromal fibrosis) occur.

The combined use of optical techniques (SHG microscopy, CP OCT and light polarization microscopy) allows assessing collagen state in the area of laser column at several levels of structural organization (Fig. 2). It led to the conclusion that the SHG image corresponds to the OCT cross-polarized image and to the image of collagen structure observed by polarization microscopy (PM) when picosirius red (PSR) staining is used.

It was demonstrated that CP OCT can visualize *in vivo* the stages of oral tissue alterations after creation of laser columns and their healing. CP OCT criteria of healing of laser columns on LPM can be the following: the presence of a layered structure in co-polarization; full restoration of signal intensity in cross-polarization.

The quantitative assessment of useful signal in CP OCT images was performed by calculating the integral depolarization factor (IDF) of OCT signal. IDF is the averaged ratio of the OCT signal in the cross-polarization to that in the co-polarization. The significant increase of IDF inside the column was found between 5 and 12 days after LPM which corresponded to the period of intensive collagen reconstruction. This is consistent with the data obtained by our group in the histomorphological study [4]. On the 90th day after the LPM treatment the IDF inside the column reached the normal (baseline) level, which means full restoration of tissue structure with no signs of scarring.

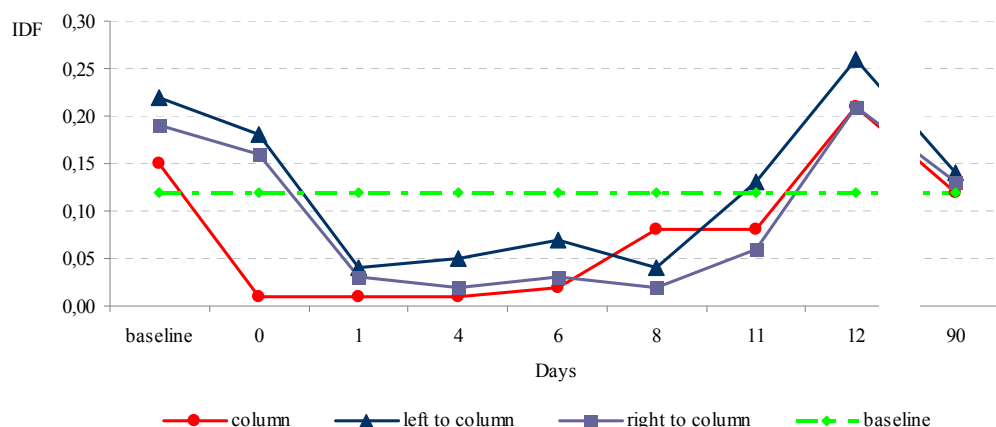


Fig. 3. Typical dynamics of integral depolarization factor (IDF) of OCT signal changes

A single treatment with laser microcoagulation is supposed to induce wound healing and regeneration response in oral mucosa, which allows considering LPM as a promising method for treating degenerative diseases of oral soft tissues.

Acknowledgements

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IN VITRO INVESTIGATION OF LASER-INDUCED HYDRODYNAMICS ON TUMOR CELLS

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Abstract. The purpose of our study was selection of a laser exposure mode to provide suppression of cancer cells *in vitro*. 1.56 μm continuous wave laser at 4.6 W output power was used to treat SKOV-3 cells. The fiber end was dipped into a culture medium and located in the center of the well at different distances from the bottom. Exposure time varied from 10 to 300 sec. The assessment of the effects was carried out by a microscopic technique 24 hrs after the treatment. It was found that treatment of cells for 300 sec at 1 mm distance to the fiber end was the most effective. Inhibition of cancer cell growth was detected. The cell amount was reduced 2-fold and 5-fold at the well center and at the periphery, respectively, as compared to the control group.

Introduction

To date, the mechanisms of laser-induced hydrodynamics in living systems are actively investigated. The examples of applications of this technology are laser engineering of cartilage, laser decompression of intervertebral cartilage, endovenous laser ablation, laser treatment of osteomyelitis etc. [1-4, 6, 7]. The formation of bubbles is known to accompany heating of gold nanoparticles by laser radiation [5]. It has been shown that the gold nanoparticles make laser treatment more local, reduce the applied power and significantly increase antitumor effect of laser hyperthermia [8].

The purpose of our study was selection of a laser exposure mode to provide suppression of cancer cells *in vitro* using laser-induced hydrodynamics. At the current stage of the work we carried out the research on the impact of laser radiation on living cells in the absence of gold nanoparticles.

Materials and methods

The study was carried out on the cancer cell line SKOV-3 grown on a six-well culture plate. A laser with a wavelength of 1.56 microns operated in a continuous mode was used for irradiation of the cells. The fiber end was immersed in the culture medium perpendicularly to the surface and located in the center of the well. The distance from the fiber end to the cell monolayer varied from 1 to 5 mm, power 0.7 to 4.6 W, and the duration of irradiation was 10 sec, 60 sec and 300 sec. After irradiation the plate was placed in a CO₂ incubator for 24 hrs. Then the cells were stained with trypan blue and counted on the inverted light microscope. To monitor the temperature during laser exposure, a computer thermograph «IRTIS», the pointwise IR thermometer and universal multimeter with a thermocouple were used.

Results

A marked inhibition of the growth of tumor cells across the surface of the wells was found. Maximum effect was achieved at a power of 4.6 W, duration of 5 minutes, and the distance to the end of the fiber of 1 mm (Fig. 1). The number of live cancer cells decreased 2-fold at the well center and 5-fold at the periphery compared to the control group without irradiation.

In order to evaluate thermal effects we monitored surface temperature during the irradiation. As is seen from Fig. 2, irradiation with a cw laser at the wavelength of 1.56 microns led to a gradual increase of the temperature of the culture medium from room temperature (25°C) to 48°C accompanied by generation of strong temperature fluctuations.

The measurement performed in the immediate vicinity of the fiber end using infrared pyrometer showed the temperature rise from 22 °C to 65-70 °C. The temperature at the periphery of the well measured by means of a universal multimeter equipped with a thermocouple increased up to 55-60 °C.

Therefore, during the selection of the mode of hydrodynamic impact on the cancer cells strong thermal effect was detected from cw laser radiation.

Another aspect of laser impact of the cells is generation of bubbles. However, at this stage it seems hard to separate thermal effects of continuous laser radiation and mechanical collapse of the generated bubbles.

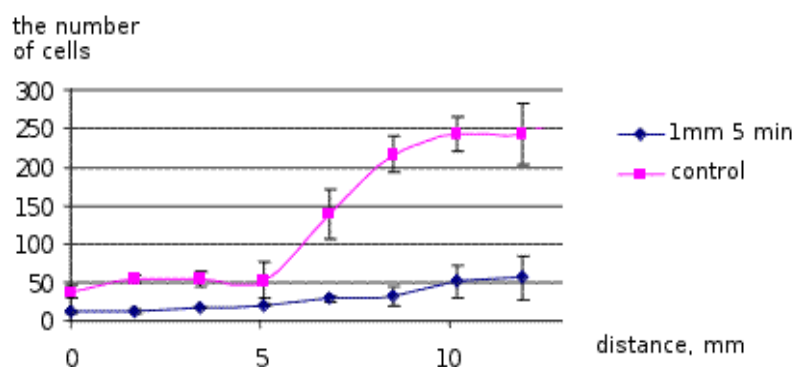


Fig. 1. The number of alive SKOV-3 cells depending on the distance from the center of the well. Blue line, laser irradiation was applied (4.6 W, 1 mm from the fiber end to the well bottom, 5 min). Pink line, control without irradiation

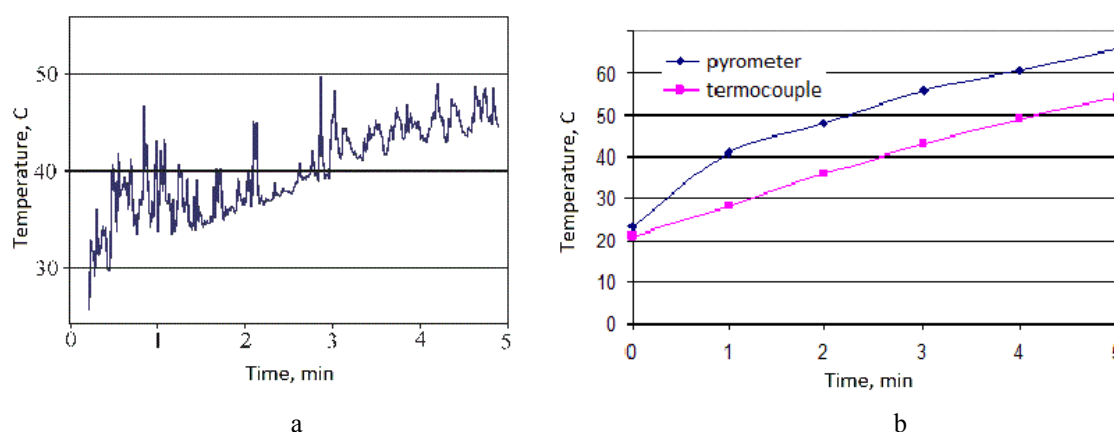


Fig. 2. Temperature increase during laser radiation (a - measured from the surface of cultural liquid by computer thermograph, b - measured at the depth of the cultural medium by IR pyrometer and thermocouple)

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SUB-ABLATIVE TREATMENT OF HUMAN HARD TOOTH TISSUES BY THE YLF: ER LASER RADIATION

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Abstract. This paper discusses the action of laser radiation with an energy density below hard tooth tissues ablation threshold on enamel, dentine and cementum (sub-ablative treatment). Sub-ablative treatment of enamel, dentine and cementum was carried out by diode-pumped YLF: Er laser radiation with a wavelength of 2.84 μm . Microhardness, acid resistance and wear resistance of intact and laser-treated hard tooth tissues were measured. It was shown that microhardness increases by 20% for enamel, by 30% for dentine, by 40% for tooth cementum, acid resistance was significantly higher for sub-ablative laser-treated enamel in comparison with intact enamel.

Introduction

In this work we discuss the action of laser radiation with an energy density below hard tooth tissues ablation threshold (sub-ablative) on hard tissues of human teeth. The effects which are initiated in hard tooth tissues by the action of such laser radiation are of great interest. The presented research can open new perspectives in esthetic and preventive dentistry. In [1] it was shown that the use of laser radiation both independently and in combination with the use of fluoride reduces enamel solubility in acid and increases its microhardness. It is also noted that dentine structures become denser under the action of laser radiation with subthreshold energy densities [2]. According to modern concepts, such effects in tissues appear due to local heating (from 100 °C to 1100 °C) which leads to the structural, chemical and crystalline changes in tissues [3–6]. At the moment attempts to use the radiation of different lasers have been made to increase microhardness and acid resistance of human hard tooth tissues. CO₂, Er-lasers, Nd: YAG, Ho-laser, and argon laser radiation was used for these purposes [1, 5]. Regardless of the fact that the results were not reproducible these investigations see perspective.

Laser treatment

In this work enamel, dentine and cementum sub-ablative treatment was carried out by diode-pumped YLF: Er laser radiation with a wavelength of 2.84 μm . We carried out a series of experiments and investigated the combinations of various parameters such as pulse duration (300–1000 μs), pulse repetition rate (3–250 Hz), fluence (0.2–2.6 J/cm²), and number of pulses (1–600 pulses). The used energy of laser radiation was below the threshold energy of ablation. Laser treatment of hard tooth tissues was carried out in non-contact mode, without water cooling. Laser radiation was focused on the tissue surface. We created textures on tissues surface. The texture is the structure of sequenced points (elements of texture) at which laser impact was achieved by a single pulse or multiple pulses at one point. The distance between centers of the texture elements was $\sim 80 \mu\text{m}$. The size of the texture was $\sim 400 \times 400 \mu\text{m}$ (5 $\times$ 5 points) for all cases.

Microhardness measuring

We measured microhardness according to Vickers before and after sub-ablative laser treatment by YLF: Er laser radiation with different combinations of parameters for tooth enamel, dentine and cementum. Microhardness meter "PTM–3M" (JSC "LOMO") was used at a load of 100 g and time of 10 s.

It was found that the highest increase in microhardness was about 20% for enamel (at pulse duration of 300 μs , pulse repetition rate of 3 Hz, pulse number at one point of 100, and fluence of 1.9 J/cm²), about 30% for dentine (at pulse duration of 300 μs , pulse repetition rate of 250 Hz, pulse number at one point of 55, and fluence of 0.6 J/cm²), and about 40% for tooth cementum (at pulse duration of 300 μs , pulse repetition rate of 250 Hz, pulse number at one point of 55, and fluence of 0.5 J/cm²).

Acid resistance measuring

We used microhardness meter "PTM-3M" (JSC "LOMO") before treatment and measured the indentations size (length of diagonals (d)) after indenter (Vickers pyramid). Indentations were obtained at load of 300 g and time of 10 s. The indentations depth (h) can be estimated based on size d :

$$h = \frac{d}{2 \operatorname{tg} \frac{\alpha}{2}}, \quad (1)$$

where α is the vertex angle of Vickers pyramid (136°).

Then we formed texture by YLF: Er laser radiation with the best parameters for increasing enamel microhardness at the site with indentations. In this fashion texture covered one half of indentations. Then we applied "Gel Etchant" (Kerr, Italy) to the sample surface containing intact and laser-treated enamel for 15 seconds in series (total time was 225 s). The sample was washed in a stream of distilled water for 15 seconds after each etching. Tooth surface was studied with "AxioScope A1 (Carl Zeiss, Germany), and photographed with "AxioCam" (Carl Zeiss, Germany) after each etching. We estimated the time at which indentations become not apparent – etching time (t). Based on this time etching velocity v_e was estimated as:

$$v_e = \frac{h}{t}. \quad (2)$$

The indentations depth (h) calculated by equation (2) was about 8 μm . It was found that etching time is 45 s for intact enamel, and 135 s for laser-treated. Thus etching velocity (v_e) for intact enamel according to equation (2) was near 180 nm/s, for laser-treated enamel - near 60 nm/s.

Wear resistance measuring

We formed texture by YLF: Er laser radiation with the best parameters for increasing enamel microhardness. After that intact and laser-treated enamel surfaces were subjected to mechanical cleaning with an electric toothbrush with abrasive toothpaste at room temperature. The duration of mechanical cleaning was for 45 minutes that is equivalent to 3 years of daily hygiene tooth brushing.

It was found that after mechanical cleaning for 45 minutes (it is equivalent to 3 years daily hygiene) with abrasive toothpaste of sub-ablative laser-treated enamel its microhardness still exceeded microhardness of intact enamel by 10%.

Conclusions

It was found that sub-ablative laser treatment leads to an increase in microhardness of enamel by 20%, of dentine by 30%, of cementum by 40%. For explanation of microhardness growth after sub-ablative laser action we proposed an enamel model which takes into account the structural peculiarities of enamel. Acid resistance was significantly higher for sub-ablative laser-treated enamel in comparison with intact enamel. After mechanical cleaning for 3 years daily hygiene with abrasive toothpaste microhardness of sub-ablative laser-treated enamel still exceeds microhardness of intact enamel by 10%.

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LASER ASSISTED IMPLANTATION OF NITIBOND PROSTHESIS

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Abstract. One problem during stapes prosthesis implantation is the fixation to the ambros. Investigation (heat conductivity, absorption, laser induced shape effects) on bulk material and stapes-implants of NitiBOND-prosthesis result in optimization of laser parameters suitable for clinical application. First clinical non-contact laser assisted implantation of NitiBOND-implants showed promising results in terms of improved hearing and reduced side effects.

Introduction

Since the introduction of stapesplasty in the 1950s [1], many innovative approaches have been undertaken in order to improve management of otosclerosis surgery [2]. New materials and prosthesis designs have found their way into the clinical routine and some disappeared after a while [3]. Nevertheless, a delicate detail problem of stapesplasty still remains the fixation of the prosthesis to the incus. Tight prosthesis fixation prevents lateralization of the piston [4] and secures long-time hearing improvement [5]. Too strong attachment can lead to incus necrosis and consequently to loss of initially restored hearing [6]. Manually crimped prosthesis requires a surgeon who has a good feel for the force which is applied. However, even experienced surgeons damage the incus, disarticulate the malleo-incudal joint, or damage the inner ear [7]. Thus “self-crimping” prostheses have been developed, produced of Nitinol, a nickel-titanium shape-memory alloy, delivered in an open form which can be put onto the incus. Heating of the prosthesis loop above a certain temperature leads to closure of the prosthesis around the incus till fixation [8]. A recent meta-analysis of stapesplasty with Nitinol prostheses has documented that use of this alloy is not inferior to established prostheses [9].

In 2010 a novel prosthesis called NiTiBOND[®] consisting of a piston of pure titanium and a Nitinol attachment loop was designed. The loop resembles a shamrock composed of four distinct contact zones and three contact-free zones which are the thermally active areas. This design should prevent thermal damage during application of energy for closure. The prosthesis is delivered with a “Thermo-Dummy” of the same properties as the prosthesis allowing for extracorporeal testing of energy parameters [7]. This study includes the investigation of the closure behaviour of NitiBOND[®] prosthesis with respect to a variety of laser parameters. A clinical study was performed to compare the post-operative hearing results after use of NiTiBOND[®] prosthesis to established prosthesis [10, 11].

Materials and Methods

In the *pre-clinical investigation* NitiBOND[®] prostheses (Heinz Kurz GmbH, Dusslingen, Germany) were fixed to a mount at the piston allowing for perpendicular irradiation onto the thermally active areas using fibres of different core diameter (400 µm, 200 µm). Different laser parameters could be investigated, e.g. wavelength, power, pulse duration, energy/pulse. Energy application, reproducible positioning and changes in prosthesis form were documented by means of a camera attached to an endoscope (Telecam[®] Endoscopic Camera, Cystoscope 0°, Karl-Storz GmbH, Tuttlingen, Germany). Qualitative evaluation was performed by comparison of the images, changes in the images could be measured (e.g. closure angle) to get quantitative results.

In a *clinical study* patients (n=61) were randomized for commercially available NiTiBOND[®] prosthesis, K-piston prostheses, or CliP[®] piston àWengen prosthesis. Posterior partial stapedectomy was performed [12]. K-piston prostheses were fixed by manual crimping, CliP[®] piston àWengen prostheses were slipped onto the incus. When NiTiBOND[®] prosthesis was chosen, a diode laser (Medilas Multi-beam, 940 nm, Dornier MedTech GmbH, Wessling, Germany) with a 400 µm laser fibre was used to apply the energy on the prosthesis loops. Pure-tone audiometry was performed according to ISO 8253-1 standard. Pure-tone average was calculated from air conduction thresholds at 0.5, 1.0, 2.0, and 4.0 kHz. Pre-operative and post-operative air bone gaps (ABG) were determined.

Results

The energy dependent closure depends on the distance between fibre tip and prosthesis surface as well as on the diameter of the used fibre. Using a diode laser emitting at 940 nm and a 400µm-diameter fibre and laser emission parameters of 40 mJ, respectively 2 W for 20 ms, a closure of about 30° could be achieved when the application was in an almost contact manner, while a distance of 1mm results in only 5° closure angle. In comparison to that, the use of a 200µm fibre results in a 10° closure angle under the same conditions. Using free beam CO₂-Laser irradiation small deviations between -2 mm and +2 mm out of the focus change focus size, thus reducing the irradiance which induces reduced closure angle. Using laser parameter like 0.5 W at 1ms the induced closure angle in the focus is about 73° while at the same laser parameters closure angle of 56° and 65° could be obtained at +2 mm respectively -2 mm out of focus deviations. Testing the laser pulse duration while leaving the applied energy constant at 40 mJ in case of 940 nm wavelength maximum closure angle of 77° could be obtained using 4 W and 10 ms. Reduced power and increased pulse duration as well as increased power and decreased pulse duration results in reduced closure angle. With respect to the wavelength dependent absorption coefficient of the material and the reflectivity specific changes in the laser energy induced closure angle could be obtained.

Clinically the use of NiTiBOND[®] prosthesis led to greater overall post-operative hearing improvement. Between the treatment groups, there was no significant difference in pre-operative ABG. Use of NiTiBOND[®] prosthesis led to a remaining mean post-operative ABG of about 11 dB whereas patients receiving K-piston prosthesis or CliP[®] piston àWengen prosthesis had a mean post-operative ABG of 13 or 19 dB, respectively. Patients in the NiTiBOND[®] prosthesis group benefitted from an ABG reduction of more than 17 dB whereas patients in the K-piston prosthesis group or the CliP[®] piston àWengen prosthesis group showed an ABG reduction of only 13.7 or 12.7 dB, respectively. In all frequencies, stapesplasty with NiTiBOND[®] prosthesis led to less remaining ABG after stapes surgery. NiTiBOND[®] prosthesis was suitable for experienced stapes surgeons as well as for ear surgeons with limited experience in stapes surgery. Remarkably, when the non-experienced stapes surgeons used NiTiBOND[®] prosthesis, they achieved a similar mean ABG reduction as the experienced stapes surgeon and even a greater ABG reduction than the experienced stapes surgeon using one of the established prosthesis types (K-piston prosthesis or CliP[®] piston àWengen prosthesis).

Discussion and Conclusion

Laser energy application for fixation of stapes implants looks a promising and easy to train technique. Due to the diversity of clinically available lasers there are a variety of parameters which should be tested separately to find a suitable set of parameters. In case of 940 nm wavelength the use of a 200 µm fibre and the application of 40 mJ with 4 W in 10 ms at a fibre to target distance of less than 1mm looks favourable for secure and safe closure of the implant without harming the surrounding tissue. Fibre assisted laser energy application looks more appropriate than using free beam laser energy application due to movements of the focus in z-direction. Laser energy impacts on the neighboured tissue at these energies should be investigated in future.

Clinically the promising audiometric results encourage further clinical use of NiTiBOND[®] prosthesis for experienced as well as non-experienced stapes surgeons. One can look forward whether this novel prosthesis will continue to allow good functional results also over a long period of time.

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CRYSTALLINE ORGANIC NANOPARTICLES – A NEW CONCEPT FOR PDT

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Abstract. The crystalline structure of Aluminium Phthalocyanine (AlPc) and other organic photosensitizers used in photodynamic therapy (PDT) can be described as stacked planar molecules. In pure water there is no fluorescence observed after excitation because of quenching effects. Nanoparticles (nAlPc) prepared of AlPc and injected intravenously into mice revealed a strong fluorescence but **only in inflamed tissue regions**. Due to the special tissue environment single molecules are dissolved from the nanoparticles and can act as sensitizers in diagnostic and therapeutic PDT. This effect will highly improve selectivity and specificity of the photodynamic diagnostic and therapy.

Materials and Methods

Crystalline AlPc raw material was fragmented to nanoparticles (NP) with a hydrodynamic size of 150nm by ultrasound. The size distribution was measured by dynamic light scattering and the samples have been provided by Biospec, Moscow. The flat molecules are stacked together [1] with quenching of the fluorescence. Under certain conditions, AlPc molecules will dissolve from the NP being fluorescent and an active photosensitizer. In [2] the authors demonstrated that inflamed tissue (skin transplants on mice) after incubation with AlPcNP showed a strong fluorescence, whereas non-inflamed skin transplants did not fluoresce. In vitro cell experiments have been performed to study the mechanism and the special conditions for the dissolution of the molecules from the nanoparticles. Fluorescence intensity was measured by microscopy (Zeiss LSM 710), as well as the fluorescence lifetimes. To measure Raman spectra, a confocal Raman microscope was used (WITec GmbH, Ulm).

Results

From experiments with different cell lines it was found that mainly macrophages are the target cells where free molecules are dissolved from the nanoparticles after incorporation into the cell. This happens in normal and also stimulated macrophages. To simulate an inflammation model, a mixture of different skin cell lines (HaCaT) and mouse fibroblast cells (L929) in combination with J774A.1 cells and also human macrophages were used. After 24 hours of incubation with AlPcNP, regions of interest (ROI) of homogeneous and identical cells were marked. The fluorescence intensity of the ROIs (Fig. 1) was taken as a measure of the amount of dissolved monomeric AlPc. The results demonstrated that the fluorescence in macrophages was dominant compared to the relative low fluorescence in the other cell types.

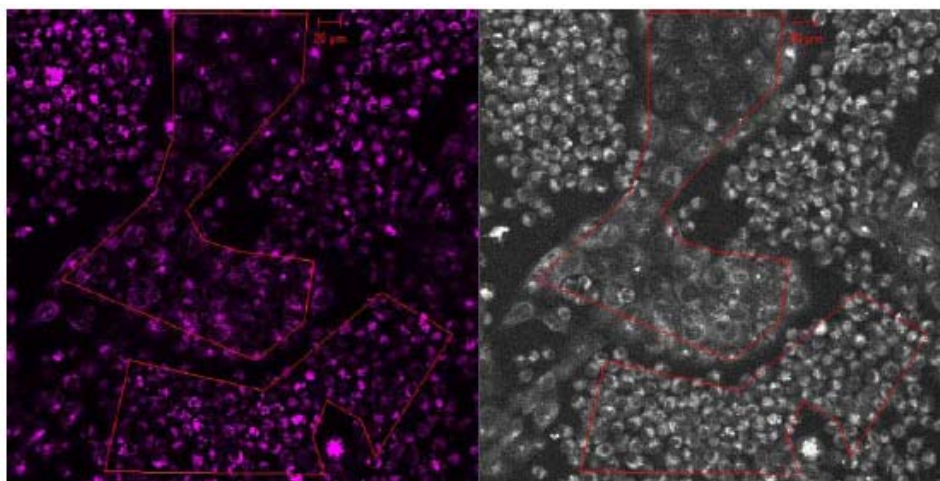


Fig. 1. Fluorescence of co-cultivation of J774A.1-cells (ROI down) and HaCaT-cells (ROI middle up)

Besides fluorescence measurements, the co-localisation of nAlPc was studied in the nucleus (DAPI), in the mitochondria (Rhodamin 123) and in the lysosomes with LysoTracker® using a laser scanning microscope (LSM 710, C Zeiss). The pictures (Fig. 2) demonstrate the intracellular sites of action of AlPcNP.

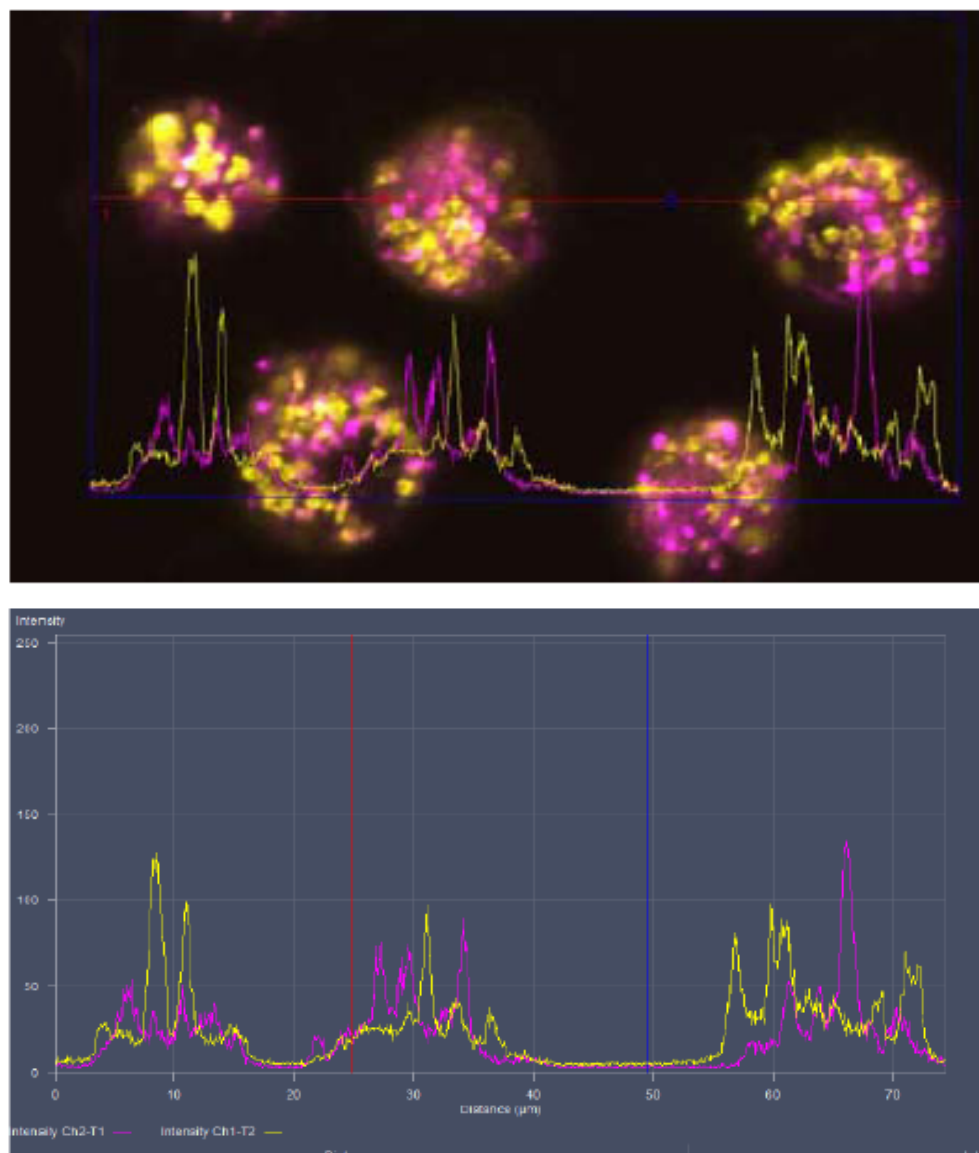


Fig. 2. Fluorescence intensity profile of co-localisation of AlPcNP (pink) and LysoTracker® (yellow)

Fluorescence lifetime measurements showed a biphasic exponential behaviour, a composition of molecules detaching from the nanoparticles and of free AlPc molecules.

Microscopic Raman experiments were difficult because of the strong background fluorescence. Further studies are planned to learn more about the microenvironment or molecules responsible for the dissolution of free molecules from the AlPc nanoparticles and the role of bacteria.

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HIGH PRECISION THz SPECTROSCOPY BASED ON QUANTUM CASCADE LASERS FOR STUDYING BIOMOLECULES AND BIOLOGICAL TISSUES

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Introduction

Medical and biological problems for THz technology involve, first of all, studying biological compounds (DNA, proteins, sugars, etc) in solid and gaseous phases, as well as liquids. Dielectric properties of biomolecules in the THz range depend on low frequency vibrations, which specify collective motion of big atomic groups, and intermolecular hydrogen bonds, thus, providing information about molecular geometry and conformational flexibility and its chemical activity. The THz technique can also be used for studying biological tissues based on dielectric function analysis. The composition of exhaled air is one of objective characteristics of physiological state and resources of humans. It is known that human exhalation is a multi-component gas mixture which contains about 600 different compounds [1]. These substances are products of physiological and biochemical processes in human organism. Therefore the analysis of exhaled air allows diagnosing some of pathology processes in human organism on the base of measuring concentrations of substance-markers.

Methods and devices

Among the variety of instrumental methods of exhaled air analysis the spectroscopic methods including the spectroscopy of THz frequency range are more prospective [1-4]. A high sensibility and resolution, as well as a possibility of noninvasive control of multi-component gas mixtures providing information about real impurity content in investigated gas and a possibility of real-time measurements are the advantages of THz gas spectroscopy method [5]. Moreover, the process of preparation of samples for measurements consists in pumping the investigated volume up to the working pressure of about 0.05 Torr. High intensity spectral lines of known molecules do not overlap, hence it is possible to take measurements at any combination of molecules. The method of THz spectroscopy is based on nonstationary effects (free damping polarization, fast frequency sweeping). Periodic switching of the phase (or frequency) of radiation interacting in resonance with the medium gives rise to the processes of transient radiation and absorption, periodic appearance and decay of the induced macroscopic polarization. The resulting transient signals are recorded and accumulated in the receiving part of the spectrometer. The value and shape of these signals are used for high-accuracy determination of concentration of components in the gas mixture.

The main scientific purpose of this work is development of a spectroscopic complex designed for studying various biological systems (biomolecules, tissues, human organism). The complex includes subTHz and THz spectrometers based on free-damping polarization and fast passing effect and operating in near and far wave zones.

The THz specific biomarker detector is designed for registration of certain gases – markers of pulmonary diseases. The device works in phase switching mode. The multiplied solid-state radiation provides a working range of 0.5–2.5 THz. The device operates at the most intensive absorption lines of exhaled biomarkers NO, NH₃, and acetone. In the radiation source of the spectrometer we use a Gunn-diode generator (97.5–117.5 GHz) together with a superlattice multiplier. The main problem in the construction of a radiation source is to obtain phase-switching in the interval $(0, \pi)$ for an arbitrary harmonic number. That is why the cornerstone of the spectrometers design involves a system of phase stabilization of the oscillator frequency in phase switching mode for arbitrary harmonic number. Phase manipulation of the Gunn generator signal maximizes sensitivity of the method and is achieved by supplying ultra short impulses to feed the circuit of the Gunn diode. The impulses are obtained by differentiation of the modulation signal which is supplied as meander to primary winding of pulse transformer through resistor. The biomarker detector can be done in “one button” configuration and with a simple interface clear for unqualified users. The preliminary investigations showed that THz biomarker detector is promising to become an optimal “electronic nose” for breath diagnostics.

The new frequency tunable generators of CW coherent radiation sources of THz radiation with frequency not worse than 10^{-8} and power $10\ \mu\text{W}$ - $10\ \text{mW}$, which meet requirements of high precision spectroscopic measurements, are developed. They are based on solid-state millimeter-range (50-120 GHz) generators with subsequent frequency multiplication and THz quantum-cascade lasers with phase stabilization and frequency control system. The developed radiation sources are used for design and development of highly precise THz-imaging system and THz spectroscopy of biological objects. The experimental setup for precise spectral measurements of DNA in THz frequency range was developed and realized.

Results

The absence of nitric oxide in exhaled air of healthy people and its appearance in exhaled air of oncopatients with cancer of lung who underwent radiotherapy are determined in clinical tests. The experimental studies of NO concentration dynamics in the exhaled air of oncological patients under radiation therapy were carried out. An increase of NO concentration (by 2-3 times) in exhaled breath of lung cancer patients after a session of radiation therapy was demonstrated.

The investigations of acetone, methanol and ethanol concentration in the breath samples of 8 conditionally healthy volunteers and 9 diabetes patients (before and after taking the medicines) were carried out. An increased concentration of acetone was found in the diabetes patients respiration in comparison with healthy volunteers. Furthermore, variation of the acetone concentration before and after the medication was revealed. At the same time, changing of the methanol and ethanol concentrations was insignificant. Moreover, the absorption line of H_2S (at the frequency of $f = 168.762\ \text{GHz}$) was detected in the exhaled breath of diabetes patient. The H_2S concentration in the exhaled breath of diabetes patient increased about 4 times in comparison with that in exhaled breath of a healthy volunteer.

The preliminary measurements of DNA spectra were carried out. The fine structure of the DNA spectrum has been investigated.

Conclusions

Application of microwave methods for development of the THz frequency range has resulted in elaboration of high precision THz spectrometers based on nonstationary effects. The spectrometers characteristics (spectral resolution and sensitivity) meet the requirements for exhaled breath analysis. The gas analyzers based on the highly precise spectrometers have been successfully applied for detection of the important biomarkers (NO, acetone, alcohols) in exhaled breath of various patients and healthy people, as well as for analysis of important biomolecules (DNA). Thus, high precision THz spectroscopy is the novel method of non-invasive diagnostics of many diseases at early stage and can be applied for screening the population for diagnostics of socially important diseases.

Acknowledgements

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Clinical Biophotonics

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OPTICAL BIOPSY OF CUTANEOUS TUMORS – FROM LABORATORY EXPERIMENTS TO CLINICAL APPLICATIONS

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Abstract. Optical biopsy tool is applied for initial diagnosis of cutaneous neoplasia, as a combination of autofluorescence and diffuse-reflectance spectroscopy of human cutaneous lesions *in vivo* and from excised tumors. Autofluorescence spectra are received using various narrow-band excitation sources in UV-VIS spectral region, and diffuse reflectance spectra are obtained using broad-band light source in the region of 400-900 nm, covering VIS-NIR spectral range. Spectra from more than 500 patients *in vivo* and about 15 different malignant, dysplastic and benign pathologies are detected and optical biopsy feasibility for differentiation of their type, stage of development and severity is evaluated. These data are used for development of methodology for optical biopsy diagnostics of skin tumours that could be introduced into clinical practice of UH-ISUL-Sofia.

Introduction

Optical biopsy is a relatively new term used in medical practice for description of spectral techniques applied for early diagnosis of tissue pathologies *in vivo*. These forms of optical diagnoses are preferable to the removal of several square millimeters of tissue surface – common in traditional biopsies – followed by delays while samples are sent for clinical analysis. In general, the predictive accuracy of optical biopsy is also better than prediction based on biopsy solely [1]. Optical biopsy apparatus in ideal would require a learning curve of several practice attempts, compared to years of training needed for some more conventional techniques [2, 3]. In the recent years, there has been a growing interest in the common use of light-induced autofluorescence and reflectance spectroscopy to differentiate disease from normal surrounding tissue – mainly for detection and differentiation of cancerous and pre-cancerous changes in human body. Light-induced autofluorescence spectroscopy (LIAFS) could be utilized to quantify differences between normal and abnormal tissues *in vivo*, providing an appropriate method for detection of pathological lesions in real time. Diffuse reflectance spectroscopy (DRS) also allows distinguishing of pathological areas from normal tissue surroundings and is usually applied for more pigmented tissue pathologies. We apply both techniques to receive useful clinical tool for optical biopsy of cutaneous neoplasia with different pigmentation levels.

Materials and methods

In this investigation we present data *ex vivo* from surgically excised cutaneous neoplasia, where excitation-emission matrices in UV-VIS spectral region are detected – to evaluate the possible sources of autofluorescence signal in the tissues investigated, as well as *in vivo* measurements on a few excitation wavelengths, of the suspicious skin lesions detected during initial visit of patients coming for diagnostics in the hospital. In cases of pigmented pathologies diffuse-reflectance spectroscopy is applied – to improve the diagnostic accuracy of lesion determination, which is suboptimal for this kind of pathologies, when only fluorescence spectra are used for diagnostics.

Ex vivo measurements were made up to two hours after surgical excision of the preliminary clinically and histologically diagnosed cutaneous lesions. The excised sample was placed in a proper liquid that supports tissue vitality up to 24 hours and transported from the hospital to the spectroscopic laboratory in thermostatic conditions. Spectrofluorimeter FluoroLog 3 (HORIBA Jobin Yvon, France) with additive fiberoptic module - F-3000 was used that allows measuring the fluorescent properties of tissue samples. *Ex vivo* point-by-point measurements were taken from the excised tumour lesions and outwards from surrounding skin. Autofluorescence using different excitation wavelengths for differentiation of tumour and healthy tissue was detected, forming excitation – emission matrix of data. Excitation applied was in the 280-440 nm region. Fluorescence emission was measured between 300 nm and 800 nm.

For *in vivo* measurements of skin pathologies, detection of lesion and surrounding normal skin fluorescence uses 365, 385, and 405 nm excitation received from several narrow-band LEDs, and 400-900 nm broad-band halogen lamp for the reflectance spectroscopy. USB4000 microspectrometer

(Ocean Optics Inc, USA) is applied as a detector and fiberoptic probe is used for delivery of the light. More than 500 patients with basal cell and squamous cell carcinoma, malignant melanoma, dysplastic nevi, keratoacanthoma, different benign skin pathologies have been investigated after initial clinical observation. Histological analysis was applied as a “gold standard” for all cases.

Results and discussion

Our autofluorescence spectra recorded *ex vivo* are from endogenous fluorophores existing in the tissue. We do not expect to have signals from co-enzymes such as NADH, NADPH, FAD and flavins, due to their fast degradation in excised tissues. We measure fluorescence signals mainly from structural compounds in the skin and its lesions. Fluorescence spectrum in the range 320-370 nm excitation at UV range of the spectrum 250-290 nm is dominated by aromatic amino acids tyrosine, phenylalanine and tryptophan, structural proteins such as collagen (excitation around 320-350 nm and emission maximum around 400-440 nm) and elastin (excitation around 290-325 nm and emission around 340 and 400 nm), their cross-links, as well as keratin are observed in the EEM data received, see fig.1a. The decrease in the fluorescence intensity of the malignant tissues is due to cancer induced destruction of collagen and elastin cross-links surrounding the tumor cells.

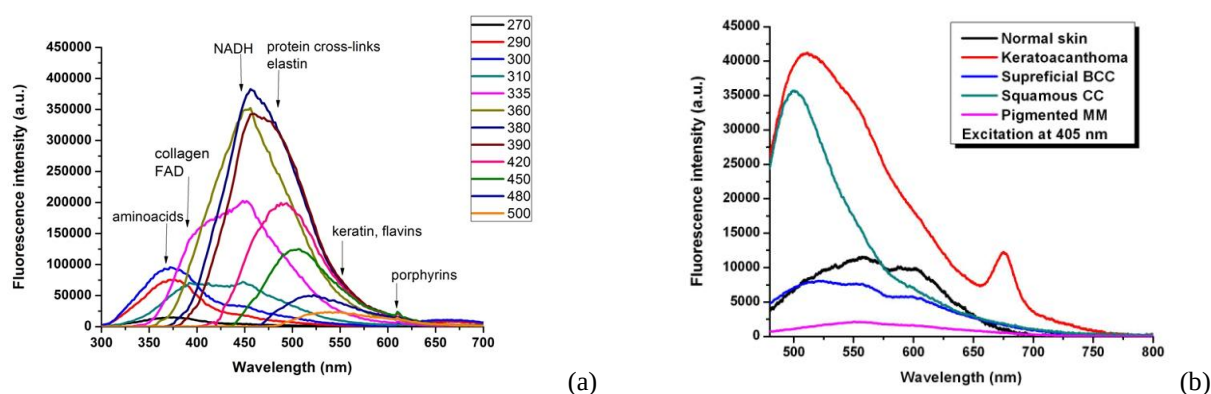


Fig. 1. (a) Fluorescence spectra of normal skin using multiple excitation wavelengths at 270-500 nm with evaluation of fluorescence origins; (b) Fluorescence spectra of different lesions using excitation at 405 nm

In the case of *in vivo* tumor measurements spectral shape and intensity changes are observed. They are specific, depending on lesion type and growth stage of the tumor, and can be applied for differentiation algorithms and cancer diagnosis, see fig. 1b. Based on our results, we have developed a significant database and revealed specific features for a large class of cutaneous neoplasia. Sensitivity and specificity observed exceed 90%, which make optical biopsy a very useful tool for clinical practice.

Conclusions

These results are obtained in the frames of clinical investigations for development of significant “spectral features” database for the most common cutaneous malignant, dysplastic and benign lesions. Our forthcoming plans include building user-friendly systems for clinical applications of optical biopsy for cutaneous tumor detection and differentiation. Recently, our group has been trying to optimize the existing experimental system for optical biopsy of skin, and to introduce it and the diagnostic algorithms developed into clinical practice of University hospital “Queen Giovanna-ISUL”- Sofia, based on the high diagnostic accuracy achieved under these investigations.

Acknowledgements

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PHOTOCHEMICAL INTERNALISATION – BASIC SCIENCE AND CLINICAL POTENTIAL

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Abstract. Photodynamic Therapy (PDT) is a technique for the local destruction of tissue with light after prior administration of a photosensitising drug. Photochemical Internalisation (PCI) is a novel technique, developed by Prof Berg and colleagues at the Norwegian Radium Hospital in Oslo. The concept is to use the principle of sub-lethal PDT to break down intracellular membranes to release biologically active macromolecules. This presentation will cover PCI studies in London on cell culture, pre-clinical *in vivo* studies on normal rat liver and transplanted cancers in the hamster cheek pouch and the first clinical trial.

Multiple drug resistance (MDR) is a problem that seriously reduces the efficacy of many chemotherapy agents. One mechanism for MDR is increased acidification of endocytic vesicles and increased cytosol pH, so weak base chemotherapeutic agents, such as doxorubicin, are trapped in endocytic vesicles. Treatments that selectively reverse this accumulation may therefore reverse the MDR phenotype. Photochemical internalization (PCI) can provide site-specific enhancement of the therapeutic efficacy of macromolecules by selective photochemical rupture of endocytic vesicles and consequent release of endocytosed macromolecules into the cytosol. Our first study evaluated PCI for release of doxorubicin from endocytic vesicles in MDR cells. Two breast cancer cell lines, MCF-7 and MCF-7/ADR (the latter resistant to doxorubicin), were studied. They were equally sensitive to PDT with the photosensitiser TPPS(2) (disulfonatedmeso-tetraphenylporphine) and light. On exposure to doxorubicin alone, the IC(50) - drug concentration for 50% reduction in colony formation was 0.1 μ M for MCF-7 and 1 μ M for MCF-7/ADR. After PCI (PDT plus administration of doxorubicin), the IC(50) concentration of doxorubicin was 0.1 μ M for both cell lines. On fluorescence microscopy in MCF-7/ADR cells, doxorubicin initially localised in granules identified as lysosomes. After PCI, doxorubicin was released into the cytosol and entered cell nuclei, as was seen in MCF-7 cells without PCI (fig. 1). Thus PCI reversed the MDR phenotype of doxorubicin resistant breast cancer cells.

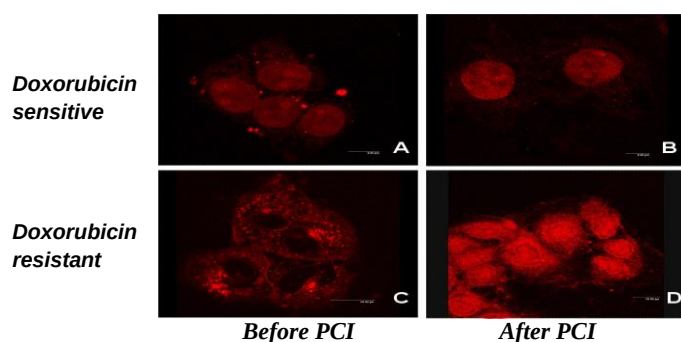


Fig. 1. Release of doxorubicin into nuclei of resistant cells after PCI, which happens without PCI in sensitive cells

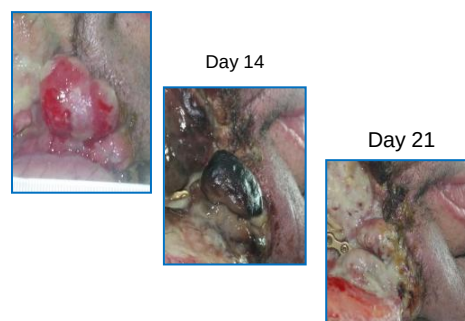


Fig. 2. PCI first in man.
Treatment of advanced cancer on the face

Pre-clinical studies in Oslo showed that PCI (PDT plus local injection of the cytotoxic agent, gelonin) enhanced the effect of gelonin alone on transplanted tumours in mice. We studied the mechanism of this in normal rats using the photosensitiser AlS2Pc (aluminium disulphonatedphthalocyanine), given 24 hours before light. After intravenous injection, gelonin was taken up and retained in lysosomes in normal liver cells, but after PCI, the lysosomes broke down to give a diffuse distribution of gelonin in the cytosol. In quantitative studies, PCI dramatically enhanced the cytotoxic effect of the gelonin on normal liver. In the absence of PCI, no effect was seen with a gelonin dose of 500 μ g/kg, but with PCI, necrosis was seen with the remarkably low dose of 5 μ g/kg (10,000 times lower than the dose at which 50% of animals died). This suggested that with PCI, less than 10 molecules of gelonin were required to kill a cell! The volume of necrosis varied with the time the gelonin was given in relation to the light, the maximum effect being seen with an interval of one hour.

Further preclinical studies in transplanted cancers in the hamster cheek pouch used the new photosensitiser TPCS_{2a}. Under optimum conditions of drug and light dose, PCI of bleomycin showed moderate tumour selectivity of necrosis.

Regulatory approval was then granted for the first PCI trial in man, recently completed, in patients with advanced head and neck cancer for whom there were no other therapeutic options. TPCS_{2a} and red light (652nm) were used for the photochemical internalisation of the chemotherapy agent, bleomycin, the dose of bleomycin being well below that used conventionally (which is associated with pulmonary toxicity). The dose of TPCS_{2a} was varied from 0.125-1.5mg/kg, the best compromise between efficacy and skin photosensitivity being at 0.25mg/kg. Blood concentration peaked at 30 mins after IV administration, but it could still be detected (by fluorescence) in skin at 4 weeks. There were no unexpected safety concerns. Although this was primarily a phase 1 safety study, there was good evidence of localised tumour necrosis in almost all patients with only minor effects in adjacent normal tissue. This study of TPCS_{2a} induced PCI of bleomycin concluded that the treatment is safe with no major adverse events. Although the study was too small to draw conclusions about the efficacy, these early results are promising and justify further studies.

Acknowledgements

This work was supported in part by PCI Biotech (Oslo), manufacturer of TPCS_{2a}.

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IDENTIFICATION OF STRUCTURAL LAYERS OF THICK AND THIN SKIN IN OCT-IMAGES

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Abstract. We performed comparative analysis of experimental OCT-images of thick and thin skin *in vivo* and Monte-Carlo simulated images based on physiological geometry of skin layers. This approach allowed us to identify structural layers of skin and to discuss optical properties of these layers. Our results show significant structural difference of thick and thin skin which should be taken into account in dermatologic OCT diagnostics.

Optical coherence tomography (OCT) is introduced as a diagnostic tool to many areas of medicine. It is widely used in ophthalmology, gynecology, and gastroenterology [1]. Despite its accessibility, skin is a difficult object for optical diagnostic techniques due to high scattering and absorption of probing light in it. When developing OCT diagnostics of skin it was shown that OCT-image of skin demonstrates layered structure with horizontal orientation [1]. Identification of these layers and their correlation with histologic images is complicated by a significant difference in optical properties and structure of the skin layers. Despite the similarity of OCT images of thick and thin skin, it appears that the optical properties of the same morphological layers vary significantly between these skin types. Current clinical OCT studies do not account for this difference [2, 3]. In this paper we propose a numerical model of OCT-images of thick and thin skin based on morphological skin structure which helps to quantify the difference in optical properties of different layers in thick and thin skin.

Thick skin is localized on palm and sole while the rest skin covering is represented by thin skin. Thick skin is characterized by the presence of the stratum lucidum and the thickness ratio of stratum corneum to epidermis close to 3, while in thin skin this ratio is about 0.3 (Fig. 1a,c). Based on similar morphological structure of thick and thin skin one would expect similar OCT-images of these skin types. However, experimental OCT-images demonstrate qualitative difference (Fig. 1b,d): epidermal layer appears as a bright OCT-image in thick skin and as a dark OCT-image in thin skin, opposite situation is observed for the upper dermis layer. The top thin bright layer in both images originates from upper stratum corneum represented by dry cells.

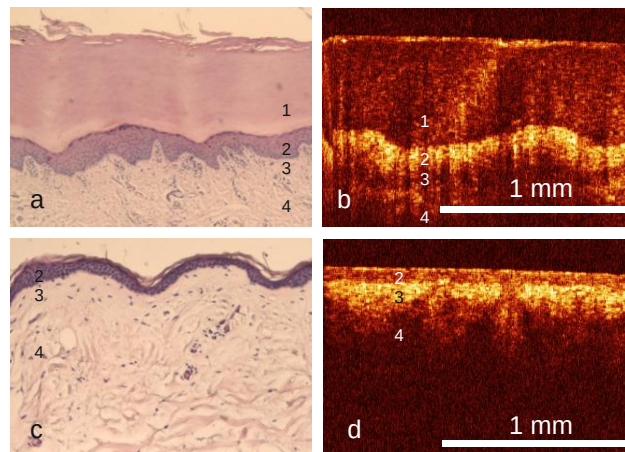


Fig. 1. Histologic and OCT-images of thick (a, b) and thin (c, d) skin.
(Layers in the figure: 1 – lower stratum corneum, 2 – epidermis, 3 – upper dermis, 4 – lower dermis)

In order to understand this difference we proposed two models for different skin types and performed Monte Carlo simulations of OCT-images [4]. The optical properties of skin layers for models of thick and thin skin based on data averaged from the literature are presented in Table 1, while morphology based geometries of skin layers are shown in Fig. 2a and 2b correspondingly.

Table 1. Optical properties of skin layers ($\lambda = 910$ nm)

Skin layer	Thin skin				Thick skin			
	μ_s (mm ⁻¹)	μ_a (mm ⁻¹)	g	n	μ_s (mm ⁻¹)	μ_a (mm ⁻¹)	g	n
Upper stratum corneum	Simulated as boundary roughness with 2 μ m amplitude				Simulated as boundary roughness with 2 μ m amplitude			
Lower stratum corneum	-				5	0.015	0.95	1.4
Epidermal layer	5	0.015	0.95	1.37	12	0.02	0.85	1.4
Upper dermis	10	0.02	0.85	1.4	7	0.015	0.9	1.4
Lower dermis	12	0.1	0.9	1.39	12	0.02	0.85	1.4

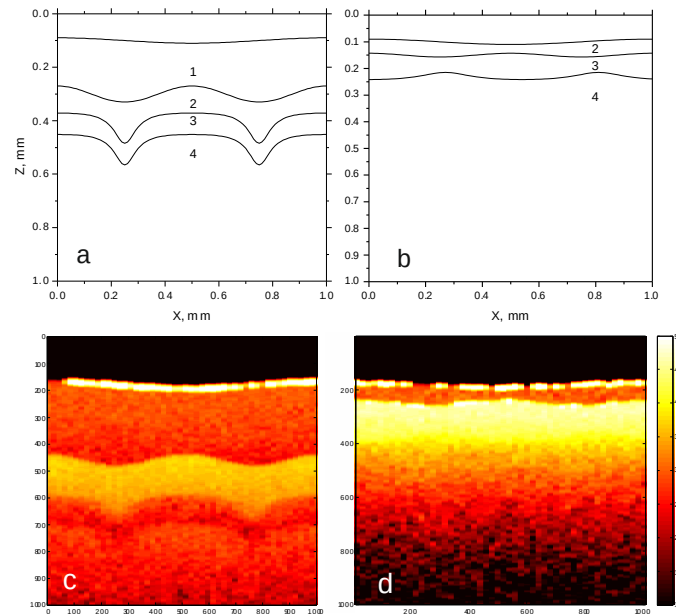


Fig. 2. Morphology based geometrical models of thick (a) and thin (b) skin employed in simulations (Layers in the figure: 1 – lower stratum corneum, 2 – epidermis, 3 – upper dermis, 4 – lower dermis); Monte Carlo simulated OCT-images of thick (c) and thin (d) skin

Figures 2c and 2d show simulated OCT-images of thick and thin skin respectively. One can see the qualitative agreement of these images with experimental ones (Fig. 1b,d). This fact confirms that the optical properties of epidermis significantly differ in thick and thin skin. One should carefully take this fact into account when performing simulations of light propagation in skin, for examples, in study of sunburn formation, as well as in development of protocols for OCT inspection in dermatology.

Acknowledgements

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MONITORING OF CLINICAL PDT WITH FLUORESCENCE IMAGING

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Abstract. Noninvasive monitoring of photosensitizer (PS) concentration in tumor and photosensitizer photobleaching in the course of PDT is of great importance. In this study we estimated a possibility of assessing these parameters by means of fluorescence imaging. Correlation between PS accumulation and photobleaching in the course of PDT and clinical outcomes (full or partial response, recurrence rate) is demonstrated.

Photodynamic therapy (PDT) is one of the modern and perspective techniques for treatment of skin tumors [1]. Damage of tumor cells is induced by a photochemical reaction originating from the interaction of a photosensitizer which is selectively accumulated in a tumor and laser radiation at a corresponding wavelength [2, 3]. The advantages of this technique are the following: localization of impact, possibility for illumination of complex-shaped areas, high factors of tolerance and safety, absence of threshold and cumulative factors limiting multiple repetitive treatments. In our previous studies we showed that the predictors of insufficient response to PDT are tumor recurrence at scar, squamous cell type of carcinoma, manifested exophytic or infiltrative component [4]. The problem of tumor recurrence after treatment should be considered from different points of view. Continued tumor growth at the periphery is connected with incorrect choice of illumination configuration. Tumor recurrence in scar most probably originates from incorrect choice of illumination dose or insufficient accumulation of photosensitizer in tumor. One of the key elements is accumulation of photosensitizer in tumor tissues (Fig. 1 a, b, c) and efficiency of its photobleaching under laser irradiation. In this respect noninvasive diagnostics of tumor borders and monitoring of photosensitizer concentration in tumor are of great importance. Ability for evaluation of these parameters in the course of PDT is promising for optimization of illumination configuration and customization of treatment.

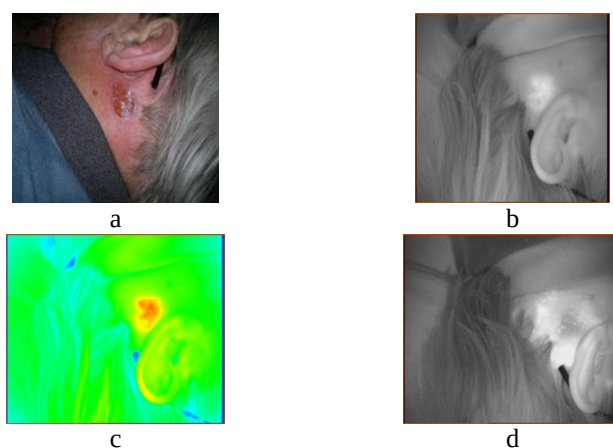


Fig. 1. Photosensitizer accumulation in tumor tissues (a,b,c) and photobleaching after PDT treatment (d)

Accumulation of photosensitizer in tumor tissue was studied in 192 patients with skin cancer at the Nizhny Novgorod Regional Oncologic Hospital. The photosensitizer photobleaching after laser irradiation was studied in 121 of these patients. Chlorine drugs (photolon and radachlorine) are used as photosensitizers. The photosensitizer dose was calculated according to patients' weight and resulted in $Me_{\min; \max} = 0.9\{0.75; 1.15\}$ mg per kg of body weight. Illumination was performed by a device equipped with a semiconductor laser emitting at 622 nm with average intensity of 0.4 W/cm^2 , minimal and maximal intensities were 0.35 and 0.5 W/cm^2 , correspondingly. Larger intensities were avoided because of manifested pain syndrome. The average dose of laser irradiation was 300 J/cm^2 . The minimal dose for small-size primary basal cell cancers without infiltrative component was 150 J/cm^2 , the

maximal dose for recurrent squamous cell carcinoma and large tumor size reached 300 J/cm². Photosensitizer accumulation in tumor tissue and photobleaching rate were evaluated by a fluorescence imaging system (Atkus Ltd, Russia). The system includes a laser radiation source (at a wavelength of 622 nm corresponding to the absorption peak of chlorine photosensitizers), a photodetection unit with a bandpass filter cutting source radiation and transmitting photosensitizer fluorescence (central wavelength 755 nm, bandwidth 90±10 nm) and a PC-based data processing unit. We monitored accumulation of the photosensitizers in tumor (using three grades: no accumulation, weak accumulation, efficient accumulation), tumor borders and agent photobleaching grade after the procedure (no photobleaching, partial photobleaching, full photobleaching) (Fig. 1d). Drug accumulation grade for two different chlorine photosensitizers is presented in Table 1.

Table 1. Accumulation grade for Photolon and Radachlorine

Drug	Photolon (N-133)	Radachlorine (N-54)
Efficient accumulation	65 out of 133 (48%)	17 out of 54 (31%)
Weak accumulation	23 out of 133 (17%)	15 out of 54 (27%)
No accumulation	46 out of 133 (35%)	23 out of 54 (42%)

Analysis of tumor response for PDT has not demonstrated significant difference depending on photosensitizers accumulation grade. The number of complete responses amounts to 90% at efficient accumulation, 93% at weak accumulation and 91% at no accumulation of the drug. However, analysis of partial response shows that the number of recurrences amounts to 5.8% at no accumulation, while at weak or efficient accumulation it amount only to 1.6%. Analysis of the photobleaching grade effect on the number of complete responses demonstrated pronounced dependence. The number of complete responses amounts to 89% at full photobleaching, 87% at partial photobleaching and 11% at no photobleaching of the drug. After full photobleaching no recurrence cases were registered, while after partial photobleaching the number of recurrences amounts to 2.4% and after no photobleaching it reaches 6.3%. The most efficient results are obtained for a combination of efficient accumulation with full drug photobleaching after treatment. For such patients the complete response is obtained in 90% cases, and no recurrences during follow-up 3-20 months were revealed.

PDT is a convenient, safe and efficient technique for treatment of skin cancer. Several factors should be taken into account when studying the problem of pathology recurrence. The key factors of photodynamic response are photosensitizers accumulation grade and photobleaching efficiency after laser illumination. Fluorescence imaging system allows one to evaluate these parameters, specify tumor borders, reveal predictors of recurrence and optimize treatment regimes. The advantages of the system are non-invasiveness, possibility for real-time evaluation of fluorescence level and accurate tumor imaging. The main disadvantage of the employed system is qualitative characterization of the obtained data. Further improvement of the data processing software for quantification of drug accumulation distribution and its photobleaching is required.

Acknowledgements

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PDT IN MANAGEMENT OF CIN AND VIN

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Abstract. Cervical cancer is the most frequent tumor in women of reproductive age. Vulval cancer also has a tendency towards rejuvenation. Both cancers are found with adjacent evidence of HPV-related precancers - cervical intraepithelial neoplasia (CIN) and vulvar intraepithelial neoplasia (VIN). The optimal method for prevention of invasive cancers consists in effective and organ preserving treatment of precancers. Photodynamic therapy (PDT) seems to be the most suitable. We demonstrate efficiency and safety of PDT in patients with CIN and VIN. We performed evaluation of morphological response and estimation of clinical results. In our opinion PDT is an alternative method for CIN and VIN with preservation of anatomical and functional integrity of organs. The III-IV grades of morphological response confirm antitumor efficacy of PDT.

Cervical cancer remains one of the most important problems in oncogynecology as it is the most frequent among malignant tumors in women of reproductive age (15–39 years) and the most frequent cause of death from oncologic diseases in developing countries [1]. In the recent decade a double increase of cases in women between 20 and 40 years old (41,21 %) is observed. This increase is especially manifested among women younger than 29 years old resulted in about 15% in the last decade [2]. Vulval cancer takes the fourth place in the structure of oncogynecologic diseases and has a manifested tendency towards rejuvenation. [3]. Both cervical and vulvar cancers are found with adjacent evidence of HPV-related precancers, known collectively as cervical intraepithelial neoplasia (CIN) and vulvar intraepithelial neoplasia (VIN) [4, 5]. The optimal method for prevention of invasive cervical and vulvar cancers consists in proper treatment of CIN and VIN, which requires organ-preserving and pathogenetically based approaches in modern conditions. Photodynamic therapy (PDT) is the most suitable for these requirements [6]. The aim of this study is determination of the efficiency and safety of PDT in patients with CIN and VIN. We performed evaluation of morphological response of CIN after PDT, clinical evaluation of PDT efficiency in CIN and VIN, and analysis of side effects and complications.

We examined and treated 12 patients of reproductive age (from 26 to 38 years old) with CIN and cervical cancer in situ, 9 patients with VIN and vulvar cancer in situ (from 30 to 45 years old) and 6 patients with recurrent vulvar cancer after surgical, combined or radio therapy. Complex clinical examination included: OCT-vulvocolposcopy, bacterioscopy, HPV-test, cytology, cervical and vulvar biopsy. In this study we used the chlorine photosensitizers (PS) in a dose of 1 mg per kilo of weight; the drug was injected intravenously with 200 ml of saline solution during 30 minutes. Laser irradiation was done at the wavelength of 662 nm. In CIN the treatment consisted of two stages: the first stage was cervical PDT and the second stage (in 30 days after PDT) – conization of cervix and histological analysis of resected portion with evaluation of tumor morphological response grade. Laser irradiation was performed in 1.5-2 hours after PS injection. Multipositional irradiation was employed: PDT was conducted at the endo- and exocervix. Quartz waveguides with cylinder diffusors with length of 3 cm were used for laser radiation delivery to endocervix. For PDT of the exocervix a macrolens providing a light spot from 1 to 2 cm in diameter was employed. Light dose was 100–200 J/cm², intensity was 250–400 mW/cm². In VIN the laser irradiation was conducted under general anesthesia (narcosis, spinal or epidural anesthesia) in 2–3 hours after drug injection. The light was delivered perpendicularly to the irradiated surface to the whole vulva and additional power was delivered to the zone of revealed tumor. The total light dose was 100–200 J/cm² for areas with VIN, 200–300 J/cm² for areas with cancer in situ, and 300–400 J/cm² for areas with local vulvar cancer recurrence. The light intensity amounted 0.7–0.9 W/cm², 1–2 W/cm², and 1.5–2 W/cm² respectively. The total light dose at the rest surface of vulva was 70–100 J/cm², with corresponding intensity of 0.5–0.7 W/cm².

HPV with high oncogenic risk (types 16, 18 etc.) was revealed in 10 CIN patient, while 2 patients were HPV-negative. After PDT the recovery was reached in 3 weeks. At this moment antiviral efficacy of PDT was estimated and full HPV eradication in 8 of 10 HPV-positive patients was identified. At

the second stage cervical resection was conducted after full epithelization of cervix (1 month after PDT procedure). Histology analysis of the resected portion allowed evaluating adequacy of conization and the grade of morphological response. As for adequacy, tumor free margins were observed in all patients. In 4 cases complete morphological response (IV grade) was reached. Grade III of morphological response (necrosis, fibrosis, single degenerative cancer cells) was revealed in 7 patients. Moderate morphological response with residual CIN II was reached only in 1 patient.

In VIN and vulvar cancer in situ the epithelization was finalized in 3–4 weeks, while in case of vulvar cancer recurrence it took 5–8 weeks. The efficacy was evaluated clinically as complete or partial response. Complete response was reached in 8 of 9 patients with VIN and in 3 of 6 patients with vulvar cancer recurrence; partial response was observed in 1 and 3 patients, correspondingly. At partial response repeated PDT procedures were conducted allowing complete response to be reached in all patients.

Note that no complications or side effects were observed in follow-up of PDT procedure. In CIN treatment high tolerance to the procedure should be noted: none of the patients complained of pains during PDT. VIN treatment is accompanied by a manifested pain syndrome which required narcosis for adequate anesthesia. The course of early post-operative period of VIN treatment was also accompanied by pain syndrome and required application of anesthetics. In our study the follow-up period for cervical pathology was 9 months. OCT-colposcopy, cytological, HPV tests and, if required, histological study (biopsy) were conducted every 3 months. One case of cancer in situ recurrence was observed in a patient with moderate tumor morphologic response which required repeated PDT. The follow-up period for vulvar pathology was 2 years. OCT-vulvoscopy, cytological and, if required, histological study (biopsy) were conducted every 3 months. One recurrence case of VIN further treated by repeated PDT procedure was observed. Repeated local recurrence of vulvar cancer appeared in two patients which required repeated PDT procedure. It is worth mentioning that the period without recurrence until the repeated PDT in these patients lasted 1 and 1.5 years respectively.

To conclude, PDT is an alternative method for CIN and VIN treatment with preservation of anatomical and functional integrity of organs which is important for realization of sexual and reproductive functions. The morphological response demonstrated in this work confirms antitumor efficacy of PDT. Manifested antiviral efficacy leads to a significant decrease in recurrence of HPV and associated CIN and VIN. For local recurrence of vulvar cancer after surgical, combined or radio therapy PDT is a method of choice. Cervical PDT does not require narcosis which provides the possibility for its application in outpatient setting. Usage of narcosis during vulvar PDT is reasonable. Absence of complications and side effects in this case may indicate the safety of the method. In our opinion, partial response or recurrence after PDT are associated with inadequate light dose, insufficient PS accumulation in tumor and manifested exophytic or infiltrative tumor component. Failures may be minimized by employing fluorescent imaging monitoring of drug accumulation and photobleaching in the course of treatment, multistage treatment strategy and combination of PDT with other methods.

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OPTICAL BREAST SPECTROSCOPY FOR MAMMOGRAPHY SURVEILLANCE STRATIFICATION

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Abstract. While mammography is credited in reducing the mortality and morbidity due to breast cancers in countries which have a national breast screening program it places an economical burden on the health care providers and on the women. The current cost in North America ranges from \$140 to 200 USD and close to 1400 mammograms are required to prevent one breast cancer related death. The major economical gains are in reducing late stage breast cancer incidence. Hence, reducing the cost while maintaining or improving the national breast cancer screening programs requires the implementation of novel prescreening technologies.

Introduction

According to a report by the OECD and Russian publications the Russian Federation is currently falling behind in providing its female population with adequate breast cancer (BC) screening, detection, diagnosis and therapy, despite spending considerable resources. Cost drivers are late stage (III/IV) BC accounting still for 37% of all tumours. This is reflected in the BC mortality increasing by 14% and 33% for women aged 25-49 years and 50-75 years, respectively between 1985-87 and 1995-97 [1]. This increasing trend is accelerating, particularly in the young group driven by the high smoking rates in this cohort, thus providing continuous pressure on the Russian Health Service overall delivery.

One reason for the high rates of stage III/IV cancer is limited access to screening as Russia has on average 3.7 mammographic units per million population. Socialized health care systems aim to have 12 mammographic units/1M population [2], so some European countries have less and mixed private/social health care systems such as the United States have 28 units per million population. In 2008 only the Moscow region had a breast cancer screening program with about 8.5 mammograms per million population [3]. In 2009 treatment of all stage I/II BC cancers cost ~\$150M USD whereas treatment of the stage III/IV cancers cost close to \$1B USD per year. A key bottleneck in expanding the mammography based screening program is the HQP training in addition to high infrastructure costs and high operating cost.

In order to render an integrated breast cancer screening program more effective and faster implementable, we propose the use of optical breast spectroscopy as a pre-screening tool to identify within the entire female population the 20-25% women who will benefit most from the available mammographic screening procedure.

Equipment setup and study groups

Initial studies use the full spectral range from 620 to 1100 nm and a total of 4 source and coaxial 4 detector positions. A total power density of $< 200 \text{ mWcm}^{-2}$ was delivered to the skin surface in compliance with the safety requirements for the exposure of skin. The collected spectra can be analyzed by principal component analysis to stratify women into highly versus not benefiting from a subsequent mammography and also average tissue chromophores concentration in the breast tissue can be computed. The current prototype devices utilize only 13 wavelength in the 645 to 1060 nm range and it utilizes only 2 source and 6 detector locations on the breast, while still permitting $> 80\%$ of the total breast volume to contribute to the optical signal.

Result to date

A first study aimed at identifying women with known physical and biological risk factors, in particular mammographic density and parity, from within the general population and we demonstrated sensitivity and specificity > 0.9 in identifying women in the top 25% risk category [4, 5]. For the considered clinical use of OBS a high sensitivity is paramount ($>98\%$) whereby reduced specificity is acceptable, whereby the available capacity of image based screening determines the attainable sensitivity.

Additionally in a longitudinal study the rate to changes in the tissue indicative for tissue aging could be determined for each participant after 6 visits as early as in their late 20th. This provides the ability to consider the rate of tissue aging a modifiable risk factor according to M. Pike [6] which can be monitored in a non-invasive manner, and potentially offers women for the first time the ability to monitor changes in their breast cancer risk over time, in particular to determine on an individual basis if risk reduction interventions such as diet and lifestyle changes will have an effect.

Additional clinical studies currently ongoing, investigate the ability of Optical Breast spectroscopy to correlate tissue aging and risk of developing BC further include a study with cohorts comprised of BrCa1/2 carriers and high risk women based on the Gail score as well as their controls and a study in women participating in hormone replacement therapy. These studies undergo currently a first interim analysis and the results will be presented at the conference.

As indicated above, PCA analysis indicated the most predictive wavelength when attempting breast cancer risk stratification equivalent on physical or demographic risk factor comparators. In the current design 8 optodes in a fixed geometry are employed to interrogate the breast tissue and a direct comparison of the predicative power vie-a-vie mammographic density a readily available risk factor. Various considerations pertaining to the clinical use of optical breast spectroscopy entering the equipment design will be presented including the need to match a cost point and autonomy of the initial risk assessment from HQP training and availability.

Conclusions

Physical prescreening based on the quantification of bulk tissue properties, rather than the generation of images, is capable of identifying women at risk of developing breast cancer and those with a high probability of harboring breast tumors from within the general population, thus increasing the benefit each women can derive from x-ray based mammography. The use of bulk tissue properties and a generic algorithm identifying women at risk reduces the burden of training a large number of additional radiologists to achieve complete population coverage for a national breast cancer screening program.

Acknowledgements

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OPTICAL METHODS FOR PREDICTION OF THE EFFECT OF NEOADJUVANT CHEMOTHERAPY OF BREAST CANCER

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Abstract. Imaging the oxygen state of breast cancers during the course of neoadjuvant chemotherapy offers the opportunity of understanding the biological changes of tumor tissue that can lead to more individualized treatment. The objective of the study was to develop a method of predicting breast cancer response to chemotherapy based on information about tumor oxygen state. Nine patients with stage II-IIIa of breast cancer were included in the study. Dynamics of tumor oxygen state was monitored with diffuse optical spectroscopy. DOS results were compared to pathologic tumor response after surgery. The increase of tumor oxygenation was a predictive criterion of a complete response, the decrease of tumor oxygenation – a criterion of a non-responding tumor.

Metabolic imaging of breast cancers during the course of neoadjuvant chemotherapy offers a possibility of understanding the biological changes that occur in the tumor in case of complete versus incomplete responses to treatment. It also affords the opportunity to develop image-based predictive biomarkers that could lead to superior individualized patient treatment. In clinic, medical oncologist should consider some prognostic factors that can influence chemotherapy effectiveness: disease stage, tumor receptor state, patient's age etc. There are no criteria allowing predicting individual sensitivity of breast cancer to chemotherapy of this very tumor. Optical methods have a potential to evaluate the dynamics of tumor metabolic state, i.e. tumor oxygen state. Indicators of tumor oxygen state were used for prediction of tumor response in [1, 2]. Diffuse optical spectroscopy (DOS) provides information on the concentration of the major tissue chromophores that reflect tumor oxygenation. It has lower cost compared to other imaging modalities, which facilitates repeated imaging procedures during treatment.

The objective of the study was to develop a predicting method of breast cancer response to chemotherapy based on information about the dynamics of tumor oxygen state. Experiments were performed on the experimental setup with parallel plane geometry and single source and detector pair created at the Institute of Applied Physics RAS (Nizhny Novgorod, Russia). Three laser fibers coupled in a single bundle illuminate the studied volume at 684 nm, 794 nm, and 850 nm. The images were acquired by simultaneous scanning of source and detector facing each other. During DOS imaging, the subjects were placed in a prone position on a padded examination platform with the breast to be imaged pendant between two plates; this setup is similar to the imaging geometry used in prone breast biopsy. The plates were moved into contact with the breast that was pressed lightly to maintain a homogeneous imaging volume during each imaging session. To place the imaging array on the breast in the same position repeatedly, the tumor was localized during the baseline DOS imaging procedure with palpation guided using positional information contained in radiology reports from prior imaging studies.

The obtained images were processed with MathCad, and concentrations of main indicators, influencing tissue oxygenation (oxygemoglobin, deoxygemoglobin, total hemoglobin and oxygen saturation) were reconstructed. The region of interest (tumor zone) was countered manually with ImageJ program according to the previous mammograms (Fig. 1). Nine patients with locally advanced breast cancer (stage II-IIIa) who underwent neoadjuvant chemotherapy as a standard-of-care treatment were included in the study. The patients received from 4 to 6 cycles of standard chemotherapy. For each participant, baseline mammograms and DOS images of the diseased breast as well as a tumor receptor state determined after needle-core biopsy were acquired prior to the initiation of the treatment. All patients signed an informed consent for the procedure. DOS imaging sessions were performed twice, the first time – before treatment beginning, the second – before the second cycle of chemotherapy.

After surgery, macro- and microscopic images of resected tissue specimens were used to categorize the subjects with complete or incomplete responses to therapy. Changes of tumor oxygenation after chemotherapy were compared to pathologic response to treatment.

Dynamics of tumor oxygen state was evaluated in 9 patients, the oxygenation trend and the patho-morphologic response were compared in 6 patients. Initial concentrations of oxygenated hemoglobin, deoxygenated hemoglobin, total hemoglobin and blood oxygen saturation that indirectly reflect tissue oxygenation [3] were evaluated.

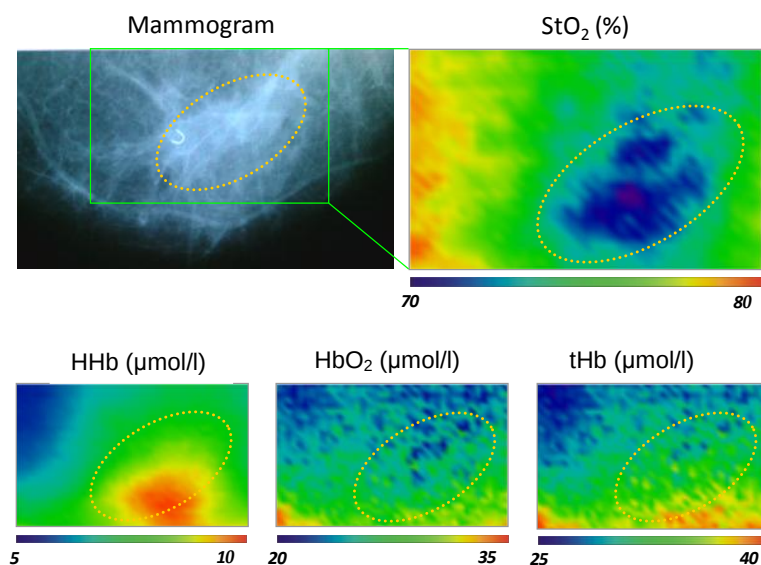


Fig. 1. An example of DOS image of diseased breast with tumor contour according to radiography

The main predictive criterion allowing evaluating chemotherapy effectiveness appeared to be a trend of tumor oxygenation changes. Complete response (necrosis, absence of viable cells) correlated with an increase of tumor oxygenation compared with its initial level after the first cycle of chemotherapy (Fig. 2a). Incomplete response correlated with a decreased or stable tumor oxygenation (Fig. 2b).

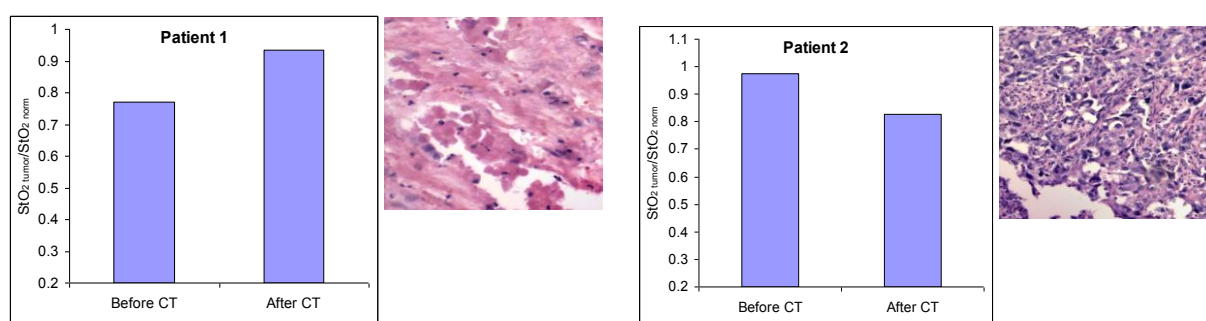


Fig. 2. Examples of the dynamics of tumor oxygenation depending on tumor response to chemotherapy.
a – complete response, b – partial response

Dynamics of breast cancer oxygenation after the first course of neoadjuvant chemotherapy appears to be a predictive factor of tumor response to treatment.

Acknowledgements

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OCT-ASSISTED MONITORING IN DIAGNOSING AND TREATMENT CONTROL

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Abstract. We report on development of two OCT-assisted diagnostic procedures consisting in OCT-monitoring of inspected tissue under physical action. In OCT-colposcopy we employ mechanical compression for diagnostics of early forms of cervical neoplasia in case of complicated recognition. We show that application of mechanical pressure leads to contracting of stratified structure in OCT images of benign alterations and does not affect the OCT-image in case of early forms of malignization. Ability of OCT detection of changes in biotissues induced by temperature action is demonstrated by way of example of OCT-monitoring of photodynamic therapy (PDT) of vulvar pathologies and cryotherapy of nasopharynx pathologies. We show that OCT is able to register typical biotissue changes induced by high temperature (PDT) and low temperatures (cryotherapy).

OCT is actively penetrating into area of visualization in clinical practice aimed at diagnostics of neoplastic and non-neoplastic lesions [1], treatment monitoring [2] and follow-up [3]. Wide introduction of this technique into practical medicine requires development of protocols for its application and criteria for interpretation of diagnostic OCT-images. One can use optical clearing techniques in order to increase diagnostic value of OCT-images [4]. Our previous studies demonstrated that mechanical compression can be efficiently employed for enhancement of OCT-image contrast in diagnostics of skin [6]. The aim of this study is to understand the effect of different physical factors on diagnostic OCT-images in gynecology and otolaryngology.

We conducted *in vivo* OCT-monitoring of different biotissues under different physical actions. The effect of controlled compression was considered in OCT detection of early forms of cervical neoplasia. The effect of high temperatures was studied in the problem of OCT monitoring of vulvar skin in the course of photodynamic therapy (PDT). The effect of low temperatures was studied in the problem of OCT-monitoring of nasopharynx mucosa in the course of cryotherapy. OCT imaging was performed using “OCT-1300U” modality (IAP RAS, BioMedTech Ltd., Nizhny Novgorod, Russia). OCT device specifications are the following: probing wavelength is 1280 nm, in-depth resolution is 15 μm , lateral resolution is 30 μm , imaging rate is 8-10 frames per second, probing depth is up to 2 mm, replaceable endoscopic probe is 2.7 mm in diameter. In it worth mentioning that construction of the OCT forward looking probe conjugated with dynamometer allows applying controlled mechanical compression to the tissue under study during OCT-diagnostics.

Figure 1 shows OCT images of cervix of a patient with HPV related cervical pathology, accompanied by acanthosis which is manifested by interpenetration of epithelium and stroma resulting in less contrasted boundary between these layers (Fig. 1a). This fact complicates interpretation of this diagnostic image and raises a doubt that the alterations in the inspected area are benign. However, application of moderate compression (130 g per probe area) leads to significant contrasting of the OCT image (Fig. 1b) which demonstrates stratified upper layers with different optical properties and manifested boundary between them allowing to interpret these alterations as benign. At the same time, when imaging the area of high grade cervical interepithelial neoplasia (CIN 3) the OCT-image remains structureless both without compression (Fig. 1c) and under moderate compression (Fig. 1d).

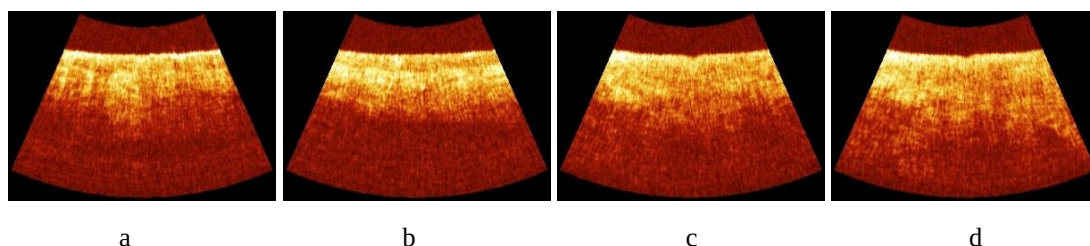


Fig. 1. OCT-image of cervical mucosa: benign alteration without (a) and with moderate (b) compression; malignant alteration (CIN 3) without (c) and with moderate (d) compression

Figures 2 and 3 demonstrate the effect of low (Fig. 2) and high (Fig. 3) temperature on the formation of OCT-images of tissue. Chronic nasopharynx inflammation is characterized by a combination of edema and fibrosis of pharynx mucosa which is manifested in OCT-images by alternating zones with high and low OCT signal level (Fig. 2a). Immediately after cryotherapy procedure homogenization of OCT signal level in upper layers is observed; we associate this effect with visually observed crystallization of water within tissue and corresponding increase in backscattering.

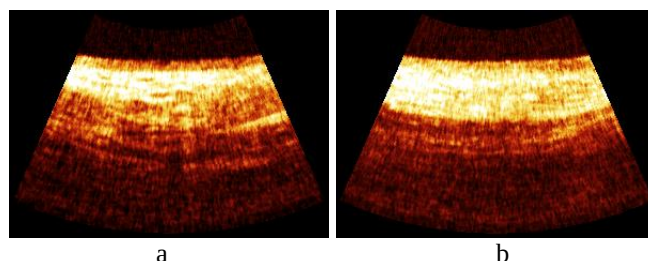


Fig. 2. OCT-images of mucosa of pharynx: chronic pharyngitis before (a) and after (b) cryotherapy

High temperature action is manifested in OCT-images in a different way. Vulva lichen, which is etiologically a chronic virus inflammation, is manifested in OCT-images as irregular shaped alternating zones with high and low OCT signal level (Fig. 3b) in contrast to normal vulvar skin (Fig. 3a). Immediately after laser action in the presence of a photosensitizer in the course of PDT, OCT-image of vulva lichen demonstrates widening of the areas with low signal level indicating development of edema (Fig. 3c). 20 minutes after the procedure the manifested edema disappears resulting in large areas with a high level signal in the upper layer. These effects in OCT-images indicate structural changes in the treated area under PDT action.

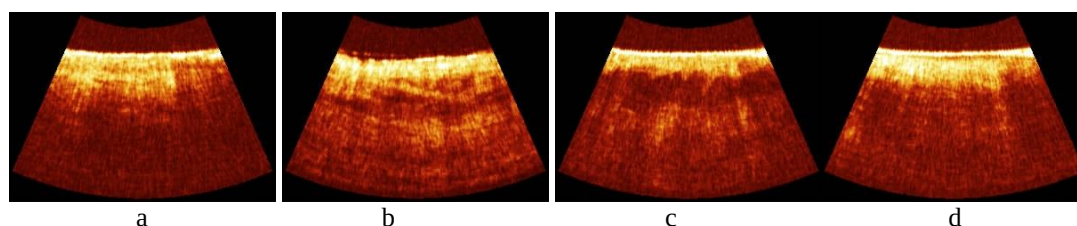


Fig. 3. OCT-images of vulva: nonaltered area (a); vulva lichen before (b), immediately after (c) and 20 min after (d) PDT procedure

Thus, we show that application of controlled mechanical compression can be employed in differential OCT-diagnostics of benign and malignant states of tissue, while OCT-monitoring allows detecting pathophysiological changes in tissues under different actions, which can be employed for evaluation of response and efficacy of treatment procedures.

Acknowledgements

The authors thank the Presidium of the Russian Academy of Sciences, the Ministry of Education and Science of the Russian Federation (project 8147) and the Russian Foundation for Basic Research (project 12-02-31191) for the financial support of this work and the hospital staff and management for the possibility to conduct this study.

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IDENTIFICATION OF MOLECULAR MARKERS FOR EARLY DIAGNOSIS OF FAT EMBOLISM SYNDROME (FES) AND OPTIMIZATION OF THE METHOD FOR PROGNOSIS, PREVENTION AND TREATMENT OF FES IN PATIENTS WITH FRACTURES

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Abstract. The study is aimed at the development of a new method for early diagnosis of fat embolism syndrome (FES) at pre-clinical stage based on the determination of the molecular markers. Identified molecular markers will help to improve knowledge of the pathogenesis of FES. Use of molecular markers will allow predicting the course of FES. Practical significance: The method of early diagnostics and prognosis of FES will enable optimization of prevention and pathogenetic therapy, as well as improvement of treatment of patients with trauma.

Every year more than 5 million people die from injuries worldwide; more than 300 thousands – in Russia. Furthermore, the death rate among able-bodied citizens from unnatural causes – accidents, poisoning and injuries in Russia is almost 2.5 times higher than in the developed countries, and 1.5 times higher than in the developing countries of Eastern Europe. More than 15-20% of death causes after trauma are from severe complications. Fat embolism syndrome is the one of these complications.

Fat embolism syndrome (FES) can be defined as a clinical condition characterized by a disturbance of lung and central nervous system functions because of the obturation of micro vessels with large globule fat. It ensues mostly after heavy injuries with fractures of long tubular bones or pelvis bones. Classical clinical picture of FES develops, as a rule, after the "light interval" followed by lung and neurological symptoms in conjunction with point bleeding on the skin. FES can occur under the guise of pneumonia, brain injury, respiratory distress syndrome in adults and other pathology that significantly increases the mortality [1, 2].

The diagnosis is made on the basis of clinical symptoms. The most commonly used set of large and small diagnostic criteria is published by Gurd [3]. In spite of the progress in the treatment of multiple and combined injuries early diagnosis of this syndrome faces many problems, especially at the stage of clinical symptoms. A wide range of studies has been conducted for the diagnosis of FES. However, none of them has 100 % specificity. Existing laboratory and instrumental methods of FES diagnosis do not satisfy the fact that changes are identified mostly just in the comprehensive clinical picture and, as a rule, only confirm the clinical diagnosis. The absence of specific markers for the early diagnosis of FES prevents the prophylaxis and early treatment of this syndrome; while timely treatment-and-prophylactic activities can prevent or reduce the decline of tissue perfusion, hypoxemia, the development of multiple organ failure, and because of this, the possible death of the patient.

Thus, the development of specific laboratory molecular markers for early diagnosis of FES on a preclinical stage is extremely necessary. Possibly, study of tissue-specific calcium-dependent regulatory proteins and protein-lipid complexes will allow identifying risk groups and pursuing a differentiated approach to the prevention and treatment of FES.

The purpose of this project is identification and functional characteristics of molecular markers, optimization of methods for prognosis, prevention and treatment of FES in patients with fractures of long tubular bones and pelvis bones.

In line with the set goals the following tasks are to be implemented:

1. Evaluate the pathogenic significance of molecular-biological markers in FES at pre-clinical stage for the patients with fractures of long tubular bones and pelvis bones.
2. Develop an express method for early diagnosis of the FES.
3. Determine the prognostic role of molecular markers in the development of FES.
4. Create a mathematical model for the prognosis of FES in patients with injury.
5. Evaluate the efficacy of differentiated prevention and treatment of FES depending on molecular markers.

Materials and methods

It is planned to examine patients with fractures of long tubular bones and pelvis bones as well as with poly trauma in the emergency ward at the Republican Clinical Hospital.

Planned medical tests for the patients: general blood test, blood test for fat globules, biochemical blood test, blood coagulation system test, x-ray of lungs, fundoscopy, analyses of molecular markers:

1) Surfactant protein D: the SP-D belongs to the collagen subfamily of glycoproteins and calcium-dependent lectins (collectins).

2) Protein S100B: S100B - calcium-binding protein, capable to form dimers.

3) Interleukin-6 (IL-6) is one of the proteins of intercellular interaction (cytokines), secreted during the inflammation.

In the patients with FES surveyed by us the level of S100B protein raised in comparison with control by 3.5 times ($p < 0.05$) on the 1st days of the post-traumatic period. Early post-traumatic changes of concentration of neurospecific S100B protein in blood serum at various heavy traumas complicated by FES along with general regularities resulted in repeated increase on the first 24 hours after trauma, and on the days to follow there appeared quantitative features depending on existence of injury of the brain.

Definition of neuroglial S100B protein in serum of blue blood in patients with fractures of the lower extremities and basin can help with early diagnostics of FES. This method is an effective and rather inexpensive way of FES diagnostics.

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PDT IN MANAGEMENT OF HPV-RELATED VULVA PATHOLOGY

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Abstract. We report on the results of PDT application in 50 patients with vulvar pathology. Clinical and morphological evaluation of the results demonstrated complete (38 cases) and partial (12 cases) response. The efficacy of repeated PDT procedures is shown. No complications or side effects were observed. The follow-up period in this study is 24 months. Methods of optical bioimaging are employed for optimization of PDT treatment strategy: OCT is used for *in vivo* diagnostics of morphological type of vulvar pathology, while fluorescent imaging provides monitoring of accumulation and photobleaching of photosensitizers. In our opinion, PDT is a radical and safe technique for vulvar pathology treatment and is a method of choice in case of local recurrence of vulvar cancer. Introduction of optical monitoring of PDT procedure may increase efficacy of vulvar pathology treatment.

Vulvar cancer takes the fourth place in the structure of oncogynecologic diseases. In 20-30% of cases the vulvar cancer progresses against the background of dysplasia preceded by various pathologic processes including HPV-related diseases [1]. Efficient treatment of these diseases prevents vulvar cancer. In consideration of a strong tendency towards rejuvenation of vulvar pathologies treatment should be not only radical, but carefully preserving urinary, reproductive and sexual functions which determine the patient's quality of life. Despite a wide variety of vulvar pathology treatment techniques (conservative methods, cryodestruction, laser vaporization, surgical treatment), their efficacy remains comparatively low. Existing methods do not ensure full elimination of local clinical manifestations of pathology, do not provide long remissions and require long treatment periods [2]. Photodynamic therapy (PDT) is supposed to be one of the most effective techniques in vulvar pathology treatment [3]. The aim of this paper is to study efficacy and safety of PDT in patients with HPV-related vulvar pathology. The tasks of the study include clinical evaluation of PDT of vulvar pathologies, approbation of optical monitoring techniques in PDT of vulvar pathologies, and analysis of complications and side effects.

We examined and treated with PDT 50 female patients with vulvar pathology aged between 25 and 83 years old, including: 15 patients with lichen sclerosus, 20 patients with squamous hyperplasia, 8 patients with VINII-III, 1 patient with cancer in situ, and 6 patients with vulvar cancer recurrence. In medical history all female patients had a long period of vulva pathology treatment by different methods including surgical treatment (vulvaectomy), radio- and chemotherapy (in cases of vulvar cancer). In this study PDT was performed employing chlorine photosensitizers (radiochlorine, photolon, photoditazyne) in a dose of 1 mg per kg of weight, the PS was injected intravenously with 200 ml of saline solution during 30 minutes. Laser irradiation was conducted under general anesthesia (narcosis, spinal or epidural anesthesia) in 2-3 hours after drug injection. The light with the wavelength of 662 nm was delivered perpendicularly to the irradiated surface to the whole vulva and additional power was delivered to the zone of revealed tumor in the course of a single PDT procedure. The total light dose was 100-200 J/cm² for areas of non-tumor pathology and VIN, 200-300 J/cm² for areas with cancer in situ, and 300-400 J/cm² for areas with local recurrence of vulvar cancer after surgical resection, radiation and combination therapy. The light intensity amounted to 0.7-0.9 W/cm², 1-2 W/cm², and 1.5-2 W/cm² respectively. The total light dose on the remaining surface of vulva was 70-100 J/cm², with corresponding intensity of 0.5-0.7 W/cm², for cancer in situ it equaled not less than 100 J/cm², with intensity of 0.5-0.7 W/cm².

As methods for evaluation of PDT results we employed clinical observation (course of post-operative period and epithelization period), vulvoscopy, cytologic and morphologic inspection. It is worth mentioning that PDT treatment of vulva is accompanied by a manifested pain syndrome which required narcosis for adequate anesthesia. The course of early post-operative period was also accompanied by pain syndrome and required application of anesthetics. In cases of non-neoplastic lesions, VIN and cancer in situ epithelization was finalized in 3-4 weeks while in case of vulvar cancer recur-

rence it took 5-8 weeks. In all considered cases we obtained full or partial response: full response was reached in 38 of 50 patients, while 12 demonstrated partial response which required repeated PDT procedures. After repeated PDT treatment full response was obtained in all patients. No complications or side effects were registered in the follow-up of PDT treatment. The follow-up period was 2 years, dynamic examinations were conducted every 3 months.

In analysis of outcomes we concluded that partial response after the first PDT procedure may originate from improper light dose, insufficient accumulation of PS in tumor, and morphologic features of pathologic area. For optimization of PDT procedure we employed optical monitoring techniques, such as optical coherence tomography (OCT) and fluorescent imaging. Preliminary results allow us to judge on efficacy of OCT in *in vivo* diagnostics of morphologic type of vulvar pathology, while fluorescent imaging provides monitoring of accumulation and photobleaching of PS.

In our opinion PDT is a radical and safe method for treatment of background and precancer vulvar pathologies. In cases of local recurrence of vulvar cancer after surgical, combined treatment or radiotherapy PDT is a method of choice. During PDT of vulvar pathologies efficient anesthesia of PDT procedure and early post-operative period is reasonable. Introduction of optical monitoring techniques into PDT procedure may increase the efficacy of vulvar pathology treatment.

Acknowledgements

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OPTICAL INTROSCOPY AS A NEW TECHNIQUE FOR SOLUTION OF TOPICAL PROBLEMS IN REPRODUCTIVE GYNECOLOGY

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Abstract. In this work we show the importance of development of novel diagnostic methods for reproductive gynecology. We developed the method combining OCT with laparoscopy and demonstrated the benefits of OCT in diagnostics of latent forms of pelvic inflammatory diseases (PID) and endometrial heterotopia not more than 0.5 cm in diameter (EH). Clinical studies including open and blind recognition tests of the OCT-laparoscopy show its high diagnostic efficacy. We report on the novel algorithm of diagnostic OCT-image processing that allows increasing the indicators of diagnostic efficacy. In future this algorithm can be employed for automated evaluation of OCT data.

A growing number of infertility and chronic pelvic pains (CPP) syndrome cases, an increasing level of latent diseases with undetermined origin in this group point out the need of novel diagnostic techniques. Pelvic inflammatory diseases (PID) и endometrial heterotopia not more than 0.5 cm in diameter (EH) are the most frequent causes of infertility and CPP [1]. Currently subacute diseases prevail among PID (60% of cases), and many of them remain unrecognized [2]. Diagnostics of EH is currently possible only in the course of laparoscopy with morphological verification of biopsy materials [3]. Introspection techniques are traditional, however, not efficient enough in diagnostics of such pathologies. [4]. Optical coherence tomography (OCT) is one of the modern techniques for introspection. In this work we combined OCT and laparoscopy which enabled a significant increase of diagnostic efficacy.

OCT imaging was performed during standard laparoscopy («Olympus Winter & Ibe GmbH», Germany) using an «OCT-1300Y» device (IAP RAS, BioMedTech Ltd., Nizhny Novgorod, Russia) with a video-frame mode. The OCT device had the following specifications: radiation wavelength 1280 nm, in-depth resolution 15 μ m, lateral resolution 30 μ m, imaging time 8-10 frames per second, probing depth up to 2 mm, replaceable endoscopic probe 2.7 mm in diameter. For preservation of pneumoperitoneum during laparoscopy and for efficient control of the flexible OCT-probe we developed a special holder. 246 female patients were enrolled in the study. The inclusion criteria: reproductive age (18-49) and indications for laparoscopy. All the patients signed the informed written consent. The study was authorized by the Ethics Commission (Protocol No. 8 of 03.11.2009).

We show that the OCT images of the isthmus part of unaltered fallopian tube are unstructured and demonstrate a moderate signal level with gradual in-depth decrease (Fig. 1a). Comparative analysis of OCT-images of unaltered fallopian tubes from 50 patients of different age at different phases of menstrual cycle demonstrated no effect of these parameters on the OCT-image of the isthmus part of fallopian tube. The OCT-images in case of PID are characterized by appearance of inhomogeneities. Comparative analysis of OCT-images and histology data has shown that prevalence of inhomogeneities and low level signal corresponds to serous inflammation with edema (Fig. 1b), while prevalence of high level signal corresponds to chronic inflammation with fibrosis (Fig. 1c). For OCT-images of endometriosis irregular-shaped areas with contrasted boundaries containing chaotically located small inclusions are typical (Fig. 1d).

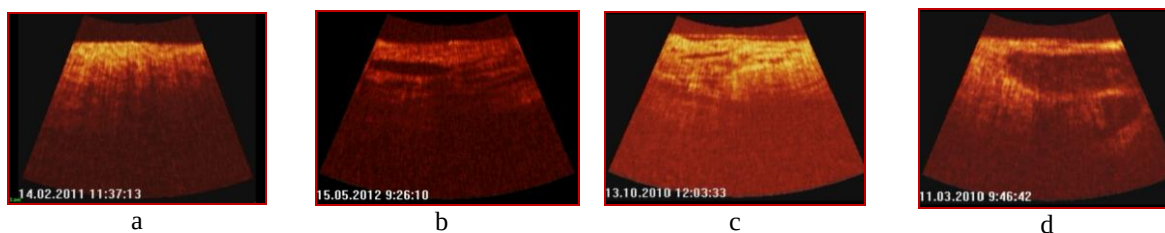


Fig. 1. OCT-images of unaltered fallopian tube wall (a), in case of serous inflammation (b), in case of chronic inflammation with fibrosis (c), and endometriosis (d). Date and time of OCT inspection are shown in the left lower corner of the image

Interpretation of the obtained OCT-images was performed based on the developed descriptive criteria as well as with the algorithm for automated evaluation [5]. For evaluation of the OCT diagnostic efficacy in diagnostics of PID we conducted a comparative open recognition test of the standard laparoscopic criteria and descriptive criteria for OCT diagnosing (29 patients with morphologically confirmed diagnosis), and a blind recognition test of descriptive (376 OCT-images) and automated (64 OCT-images) criteria for OCT diagnostics. The results are presented in Table 1.

Table 1. Comparative analysis of diagnostic efficacy of laparoscopy and OCT-laparoscopy

Factor	Laparoscopy (open recognition test), %	OCT-laparoscopy (open recognition test), %	OCT-laparoscopy (blind recognition test, descriptive criteria), %	OCT-laparoscopy (blind recognition test, automated criteria), %
Sensitivity	43.5	95.7	90	96
Specificity	66.7	83.3	81	100
Diagnostic accuracy	48.3	93.1	88	96

High interobserver agreement kappa 0.63 with a confidence interval of 95% (0.5082; 0.7453) allows concluding that the OCT-laparoscopy is objective.

Thus, our results demonstrate high potential of optical introscopy in revealing “silent” alterations in fallopian tubes and distinguishing inflammation type which enable decreasing the number of unrecognized pathologies and optimizing treatment strategy. In diagnostics of endometriosis OCT can substitute biopsy in the cases which require morphological verification, however, the biopsy procedure is dangerous, contraindicative or undesirable (area of vessels, uterosacral ligaments, fallopian tubes).

Acknowledgements

The authors thank the Presidium of the Russian Academy of Sciences and the Ministry of Education and Science of the Russian Federation (project 8147) for the financial support of this work and the hospital staff and management for the possibility to conduct this study.

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OPTICAL COHERENCE TOMOGRAPHY IN DIAGNOSING NON-NEOPLASTIC LESIONS IN ENT

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Abstract. The problem of diagnostics of non-neoplastic lesions in ENT originates from a wide variety of morphologic types of pathologies and prevalence of latent processes. At the same time understanding and recognition of etiological and morphological type of diseases is essential for the correct selection of medications and for successful treatment outcomes. In this paper we propose application of OCT for diagnostics of chronic rhinitis and pharyngitis. We show principal ability of OCT to detect morphological alterations in mucosa of nasal cavity and pharynx typical for chronic inflammatory process and to dynamically monitor changes of mucosa in the application of medications and cryotherapy. In our opinion, OCT shows high potential for noninvasive differential diagnostics of different forms of chronic inflammatory diseases of ENT and in real time monitoring of treatment.

Inflammatory diseases of upper airways, rhinitis and pharyngitis are among most frequent causes of office visits to primary care physicians. The term "rhinitis" denotes nasal inflammation causing a combination of rhinorrhea, sneezing, congestion, nasal itch, and/or postnasal drainage. As for rhinitis there are many clinical forms of this pathology: allergic and nonallergic (NAR), mixed and persistent rhinitis. Some of these subtypes are less well understood and less often diagnosed [1]. The heterogeneous nature of this group of disorders complicates choice of treatment strategy and requires more precise diagnosis [2]. Pharyngitis is a group of pathologies of different origin leading to various pathomorphological alterations and requires different treatment approaches [3]. Consequently, development of novel diagnostic tools for this pathology is of importance. Optical coherence tomography (OCT) proved to be a perspective tool for diagnostics of both neoplastic [4] and non-neoplastic [5] lesions in ENT. In the literature we found reports on application of OCT for imaging mucosas of the upper airways [6], including mucosa of nose [7]. The aim of this work is to study the ability of OCT to image alterations of mucosa of nasopharynx, typical for chronic inflammatory diseases.

We conducted the pilot study for 10 patients with chronic inflammatory pathologies of nasopharynx. It is worth mentioning methodological points affecting the complexity of this study. First of all, for development of criteria for pathology recognition in OCT images one should determine typical features of OCT-images of norm. Unfortunately, it is extremely difficult to find a volunteer with absolutely unaltered mucosa of nasopharynx, in this respect, we examined volunteers from the group of researchers with relatively normal nasopharynx. Another complication consists in impossibility of obtaining biopsy material for comparative analysis of OCT and histology data, because excision studies of nasopharynx are not conducted in case of inflammation. OCT imaging was performed using "OCT-1300U" modality (IAP RAS, BioMedTech Ltd., Nizhny Novgorod, Russia). OCT device specifications are the following: probing wavelength is 1280 nm, in-depth resolution is 15 μm , lateral resolution is 30 μm , imaging rate is 8-10 frames per second, probing depth is up to 2 mm, replaceable endoscopic probe is 2.7 mm in diameter.

Figure 1 demonstrates OCT-images of mucosa of inferior nasal concha in norm and in case of chronic rhinosinusitis before and after application of adrenalin. Adrenalin is chosen in this study as vasoconstrictor which is widely used in otolaryngology. In case of relative norm (Fig. 1a) one can distinguish upper epithelial layer and lower stromal layer represented by rich structural features, such as round or oval inclusions presumably corresponding to blood vessels. The overall OCT-signal in this case is moderate. Rhinosinusitis (Fig. 2a) significantly affects the OCT-image: the average level of the OCT-signal significantly rises and dark elongated areas corresponding to edema appear. Application of adrenalin induces significant vasoconstriction manifested in OCT-images by thinning of stromal layer, loss of contrast between epithelium and stroma (Fig. 1 c,d) and extinction of edema effects and decrease in imaging depth in case of rhinosinusitis (Fig. 1d).

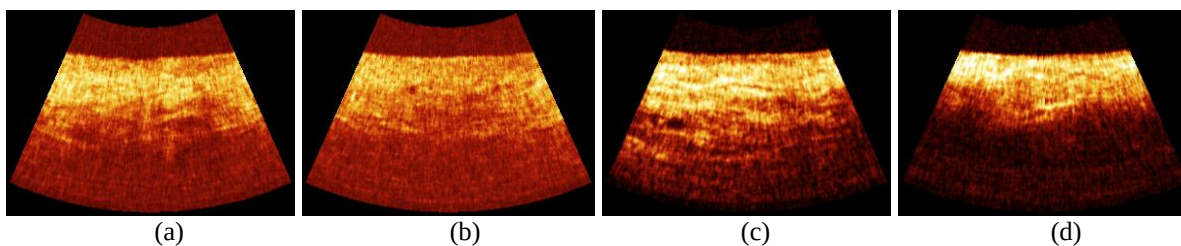


Fig. 1. OCT-images of mucosa of inferior nasal concha: relative norm (a), relative norm after local adrenaline application (b), chronic rhinosinusitis (c), and chronic rhinosinusitis after local adrenaline application (d)

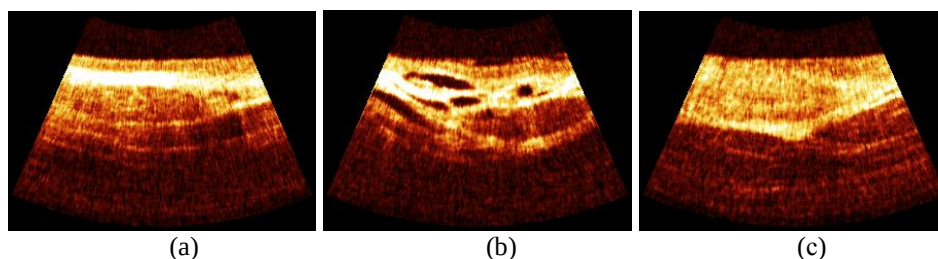


Fig. 2. OCT-images of mucosa of pharynx: relative norm (a), chronic tonsillopharyngitis before (b) and after cryotherapy (c)

Figure 2 shows OCT-images of pharynx in norm and pathology. The OCT image of relative norm (Fig. 2a) fully corresponds to morphological structure of pharynx: the upper layer characterized by moderate OCT level corresponds to stratified squamous epithelium, while the lower layer with high OCT-signal layer corresponds to *lamina propria* with high content of elastin fibers; in submucous layer glands and lymphoid elements are manifested in OCT-images by irregular shaped areas of different size and signal level. Inflammatory alterations are accompanied by pronounced edema of tissues leading to disintegration of morphological structures (Fig. 2b). Cryotherapy is accompanied by water crystallization manifested in OCT-images by disappearance of low-signal areas and balancing of signal level in upper layers (Fig. 2c).

Significant variations of OCT-images of nasopharynx in norm and pathology from patient to patient complicate interpretation of diagnostic OCT-images and require development of algorithms for numerical processing of OCT-images allowing to increase diagnostic accuracy of the technique.

In our opinion, OCT has a potential for noninvasive differential diagnostics of different forms of nasopharynx chronic inflammatory diseases and real time monitoring in course of treatment.

Acknowledgements

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PROGRESS IN FLUORESCENCE DETECTION AND PHOTODYNAMIC THERAPY FOR THE MANAGEMENT OF MALIGNANT GLIOMA

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Abstract. The use of 5-aminolevulinic acid (5-ALA) induced protoporphyrin IX (PpIX) for fluorescence guided resection (FGR) and photodynamic therapy (PDT) in malignant glioma will be reviewed. Progress has been achieved in clinical studies to more precisely define the role of FGR for the detection of glioblastoma (GBM) and other primary and metastatic brain tumours. The ultimate goal, however, is the destruction of GBM by PDT. Some intriguing long time survivors stimulate research in PDT related immune response and cancer stem cell destruction.

Fluorescence Guided Resection (FGR)

Fluorescence guided resection (FGR) is increasingly applied during open surgical glioblastoma removal [1, 2]. EU approval has been obtained in 2007, approval trials are under way in South Korea, Japan, Australia and USA. It has shown a performance comparable to intra-operative MRI and has contributed to the end of the dispute, whether a “safe gross total resection” can significantly increase survival time by showing yes, it can [3]. 5-ALA has been used to detect foci of anaplastic tissue within lower grade gliomas and for brain metastases of other tumors it has been found helpful in approximately half of cases [4]. FGR has also successfully been tried in a number of spinal cord tumors.

An intriguing approach is to use fluorescence guidance already during taking the initial stereotactic biopsy [5]. This would not only enable obtaining a suitable tissue sample from vital tumor with a very high reliability, but also quantifying the PpIX concentration in order to judge, whether the patient is a potential candidate for PDT.

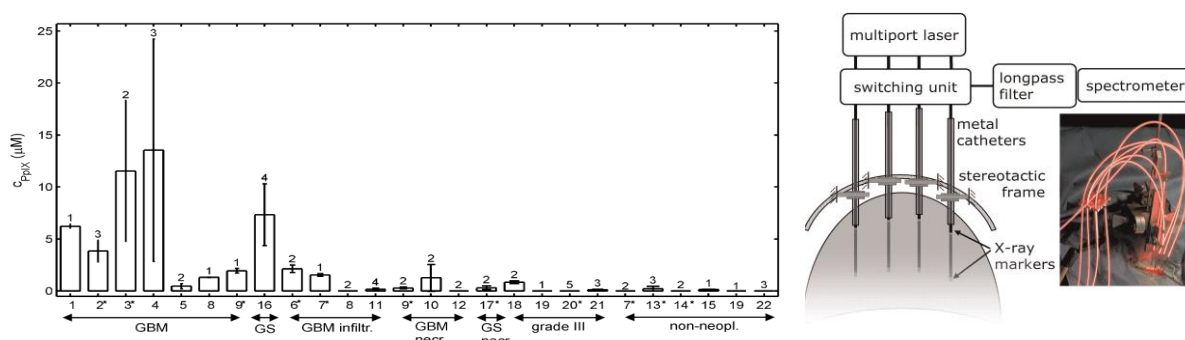


Fig. 1. Left: concentrations of PpIX found in tumour tissue during FGR with 20 mg/kg bw 5-ALA. Primary tumours are indicated by *. Indicated are also the number of tissue samples analyzed and the corresponding standard deviations. Right: Principal setup for iPDT of brain tumours with real time spectroscopic monitoring. The switching unit controlled which fibre was employed as a detector fibre

Photodynamic Therapy (PDT)

PDT for GBM treatment has a long history, but no real breakthrough can be stated until now. Most experience has been obtained with porfimer sodium (Photofrin) and promising results were obtained, but the survival benefits and side effect profiles did not lead to a broader acceptance of the procedure. Since FGR with 5-ALA is available, only few groups have explored the PDT potential of 5-ALA induced PpIX. In most cases, Photofrin has been used in addition, not trusting in a sufficient phototoxic potential of the accumulated PpIX. We have performed a controlled study and several individual treatment attempts to apply interstitial 5-ALA-PDT with stereotactically implanted radial diffuser fibers [6]. From quantitative assessments of PpIX tumor concentrations, we learned that PpIX concentrations can vary extremely from patient to patient (Fig. 1) [7]. We have therefore recently implemented PpIX-fluorescence measurements during the interstitial PDT irradiation (Figs. 1, 2). Although

this type of fluorescence assessment has no good spatial resolution, we could discriminate responders from non-responders by the intra-operative fluorescence and photobleaching (Fig. 2) [8].

Apart from direct phototoxicity, one should keep in mind the well known immune response PDT can induce. Inevitable inhomogeneous drug and light dose distributions might be advantageous to induce a competent immune response. Especially heat shock proteins, such as HSP-70 are induced by a sublethal PDT [9, 10].

Finally, one should consider the role of cancer stem cells. Although PpIX has been found to be a substrate of ABCG2, one of the multidrug resistance proteins associated with stemness, it is not finally clarified whether such cells would be resistant to PDT. If so, one might overcome their resistance by a transient blocking of this membrane transporter by approved pharmaceuticals.

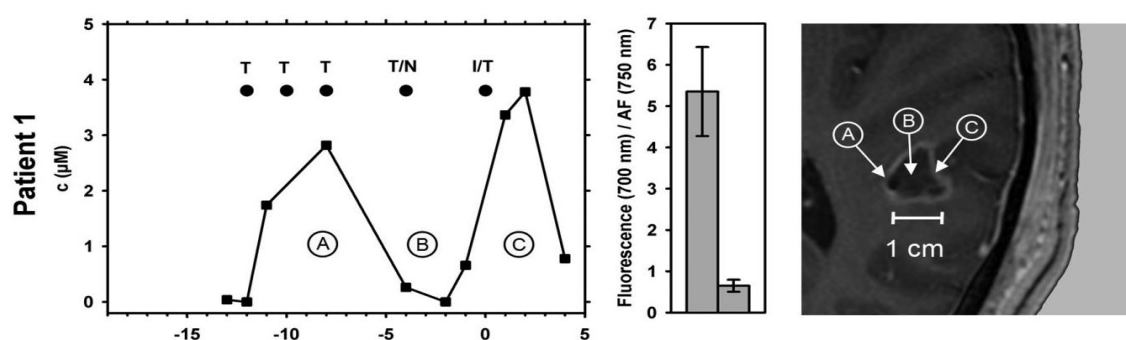


Fig. 2. Example of 1 of 5 patients reported in [8]: Absolute PpIX concentrations determined by chemical extraction along the biopsy trajectory prior to PDT. The tumour depth 0 mm corresponds with the distal tumour point along the trajectory. Areas of vital tumour (T), necrosis (N) and infiltrating tumour (I) as determined from the H&E stained biopsies (filled circles) are also indicated. The bar graph shows the mean fluorescence signals relative to the autofluorescence measured between irradiation fibres before and after the treatment session. Right: contrast enhanced NMR image pre-PDT

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In memoriam Dr. Ann Johansson, participant of TPB 2009, died 2011 during mountain climbing.

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IMPROVING THE FLUORIMETRIC METHOD OF VITAMINS A AND E DEFINITION IN BLOOD AND ITS APPLICATION IN GYNECOLOGY

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Abstract. This work describes the results of biochemical and clinical investigations concerning the influence of concentrations of vitamins A and E on female reproductive system. The method of vitamin concentration definition in blood has been improved using spectral techniques. It was shown that new maxima of absorption are generated in the UV region with increasing vitamin concentration. It can be connected with generation of vitamin associates. Similar dependences were also shown in fluorescence spectra. Finally, determining vitamin status of patients for diagnostics of female reproductive system can be based on the results of blood spectral analysis.

Introduction

Vitamins A and E are basic components of nutrition regulating biochemical and physiological processes by means of activation of fermentative reactions. According to the data of the Institute of Nutrition of the Russian Academy of Medical Sciences, prevalence of hypovitaminosis is 22% for tocopherol and 70% for carotene. Deficit of these vitamins can lead to insufficient production of gender hormones and to abnormality of the reproductive function in the organism.

In spite of the topicality of hypo- and avitaminosis, there are no accurate methods of determining vitamin concentration in patient's blood. The spectral methods of biochemical analysis are based on plotting a calibration curve of changing vitamin fluorescence intensity at one wavelength as a function of its concentration. Fluorescence intensity, however, depends on factors such as intensity of light source, temperature, moisture, etc. It is difficult to obtain accurate quantitative results even at device calibration.

This work is dedicated to enhancement of accuracy of spectral techniques for assessing concentration of vitamins A and E in blood for further application in gynecology.

Objects and methods of research

The objects of research were standard solutions of α -tocopherol (1 mg/ml) and retinol acetate (0.34 mg/ml) in hexane. A series of vitamin solutions were prepared in experiments by means of serial dilution. Hexane was as a solvent because of its non-polar structure. Hexane is often used in biochemical investigations.

Absorption spectra, fluorescence excitation and fluorescence spectra were measured on spectrofluorimeter SOLAR CM-2203 operating in photometric and fluorimetric mode in turn. The fluorescence excitation spectrum was recorded at the wavelength of 370 nm for vitamin E and 335 nm for vitamin A. The fluorescence excitation spectra in the UV region were measured at the wavelength of 410 nm for α -tocopherol and 460 nm for retinol acetate. In all the experiments the free-prepared solutions were used only.

Results

It was shown that new fluorescence maxima are generated with an increase of vitamin concentration. The spectrum becomes multicomponent. One fluorescence band of fluorescence is at 413 nm in case of α -tocopherol with concentration 34.8 nM. The increase of the concentration of vitamin E up to 0.176 μ M leads to generation of the second maximum at 450 nm. The subsequent rise of α -tocopherol concentration leads to generation of new maxima at 405, 428 and 454 nm; however, the band at 413 nm remains intensive. Fluorescence spectra of retinol acetate have similar dependences.

These regularities stimulate investigation of absorption bands in the UV spectral region. For all the used concentrations one absorption band is at 270 nm for α -tocopherol and at 280 nm for retinol acetate with optical density of about 0.3. Moreover, with the increase of vitamin concentration tens of times, the optical density changes insignificantly. Consequently, for investigation of solutions we measured the fluorescence excitation spectra analogous to the absorption spectra, as the fluorescence spectra are more sensitive to solution components.

It was shown that an increase in vitamin concentration results in the decay of the absorption maximum of solutions in the far UV-region and in generation of new maxima in the near-UV region, which may be connected with vitamin molecules associates.

Maxima in the UV-region characterize the presence of specific functional groups in the studied compound. Generation of new bands in the UV-region allows hypothesizing that vitamin molecule associates – dimmers and, probably, trimers are synthesized. However, IR-spectra are the most informative for this case. They characterize vibrations of each type of chemical bonds. Their analysis confirms the hypothesis.

The IR-spectroscopy studies showed that no significant changes of spectra occur in the region less than 1500 cm^{-1} . A pronounced decrease of intensity of the band characterizing coupled oscillations of the *O-H* group occurs with an increase of concentration. The serial concentration of solutions leads to increased oscillations at the bands characterizing the presence of free- *O-H* groups and hydrogenous connection bond.

Assessing the concentration of vitamins A and E in patients' blood was based on the results of the spectral analysis aimed at evaluating the state of female reproductive system. 46 females aging from 18 to 26 years old participated in the study. Their blood sampling and vagina contents inoculation in the nutrient media (MRS and blood agar) were carried out. It was found that the menstrual dysfunction occurs more often in the case of hypovitaminoses A and E. Intensity of abnormality correlates with hypovitaminosis severity in the organism [1]. For females with deficit of tocopherol the disbiotic abnormalities of vagina biocenosis were detected which were manifested as deficit of lactoflora and rising of conditionally pathogenic flora quantity. Analogous changes were more intense in the group with combined deficit of vitamins A and E [2].

The spectral and microbiological studies showed that hypovitaminosis A and E correlate with each other. Hypovitaminosis A leads to hypovitaminosis E and vice versa. The disbiotic states of lower reproductive tract were diagnosed for 36.4% cases in the control group in which vitamin concentration was normal. At the same time, in the case of hypovitaminosis, it was diagnosed more than twice more often 2 times (76% and 83% for isolated and combined hypovitaminosis, respectively).

Thus, the correlation between spectral and clinical has been presented in this work. The method of calibration curve construction cannot be applied for vitamin concentration assessment in blood. The presence of possible vitamin associates with an increase in their concentration can lead to mistakes in biochemical analysis. During quantitative measurements fluorescence spectra and/or fluorescence excitation spectra need to be registered. This approach was also described in our previous work [3]. Assessing of vitamin status of patients and prognosis of available abnormalities of female reproductive system can be based on results of changes of spectral-luminescent properties of vitamins.

Acknowledgements

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***Biophotonics
in Stem Cell Research
and Developmental
Biology***

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CONFOCAL IMAGING, OPTICAL PROJECTION TOMOGRAPHY AND FOLLOWING 3D-RECONSTRUCTIONS OF WHOLE-MOUNT STAINED VERTEBRATE EMBRYOS APPLIED FOR STEM CELL RESEARCH

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Abstract. Neural crest cells represent an extremely multipotent migratory population of embryonic stem cells originating from dorsal neural tube shortly after gastrulation. Since these cells are highly migratory and give rise to dozens of other cell types, three-dimensional reconstruction of embryos with visualized neural crest cells and their progeny is highly desirable. To achieve an excellent degree of neural crest visualization in the entire developing body we label these cells with genetic tracing using *Brainbow* multicolor reporter. This approach together with 3D reconstruction enables demonstrating clonal migrations and mixing between progeny of individually labeled neural crest cells in the developing head and trunk of a mouse embryo. Additional recent advancement of this technology was brought by the introduction of *Scale* reagent into our work, which yielded better optical transparency of a sample without denaturing fluorescent proteins and, thus, deeper imaging solutions. In parallel with genetic tracing techniques we also apply fluorescent whole-mount immunohistochemistry using a variety of antibodies and their combinations. Apparently, high concentrations of DMSO in all antibody-containing solutions significantly improve the efficiency of antibody penetration allowing us to image and reconstruct stem cells in whole mouse embryos up to embryonic day 12. In addition to this, application of Tyramide Signal Amplification (TSA) reaction increases the sensitivity and specificity of our whole-mount staining especially in younger embryos. Finally, we demonstrate how IMARIS software suits most of our needs related to 3D-reconstruction in fast and convenient way including segmentation, generation of surfaces and necessary measurements on reconstructed 3D-objects.

Recently we discovered an entirely new phenomenon in developmental biology – targeted recruitment of neural crest-like stem cells from the pervasive peripheral nerves. For example, we demonstrated that the majority of melanocytes – our pigment cells, are born from peripheral glial cells [1, 2]. Current opinion holds that sensory innervation [3] of different locations in the body has a minor or no role in making a structure of an organ during development and adulthood. On the contrary, our preliminary data strongly suggest that sensory nerve contributes multipotent neural crest cells to a number of locations known to share developmental contribution from neural crest lineage. Our hypothesis implies that nerve-associated glial cells can be recruited from the nerve by unknown molecules presented inside the skin and internal organ environment, and that these recruited cells are capable of producing local differentiated progeny. For example, utilization of such developmental strategy could lead to the complexity reduction in path-finding programs involved in migratory behavior of neural crest-derived precursors. This is a new developmental principle, which is probably widely implemented also during regeneration of different peripheral compartments or during the maintenance of tissue homeostasis.

To experimentally address our hypothesis we applied several innovative and powerful approaches: we use advanced genetics tracing with multicolor reporters, completely novel unconventional individual cell transcriptome analysis, transgenic mice with cell type-specific modifications in signaling, microsurgery and grafting, and, finally, 3D-imaging of developing embryonic structures. We utilize a novel type of genetic tracing, which involves recently generated *Rosa26-stop-Confetti* (*Brainbow 2.1*) reporter consisting of four different fluorescent proteins (nuclear GFP, cytoplasmic YFP, cytoplasmic RFP, membrane CFP) cloned in a tandem and spaced by loxP sites. This allows random recombination and activation of the only one fluorescent protein upon Cre-mediated recombination. Thus, *Confetti* fluorescent reporter (obtained from Prof. Hans Clevers, Hubrecht Institute, Netherlands) enables exceptional clonal fate mapping *in vivo* since labeled clones and their progeny might be labeled with individual colors. Application of 3D visualization techniques allowed reconstruction of innervation with immobilized neural crest-like immature glial cells and also their progeny in the skin and in cranio-facial locations.

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FLUORESCENT IMAGING MODALITIES FOR MESENCHYMAL STEM CELL-TUMOR TROPISM

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Abstract. Mesenchymal stem cells play an important role in tumor pathogenesis. In this study we investigated various models of the interaction of tumor and stem cells using two methods: *in vivo* imaging and *ex vivo* confocal laser scanning microscopy. Two models were used in the experiments. In the first one, the interactions between adipose-derived human adult stem cells and the organism of nude mice at different stages of tumor growth were investigated. In the second model, GFP (+) stem cells bone marrow was administrated in irradiated mice with transplanted tumor. It was shown that stem cells of different origin integrate in the animal organism with transplanted tumor and can be identified by different methods.

LASER ANALYSIS AND MICROMANIPULATION OF PREIMPLANTATION MOUSE EMBRYO

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Abstract. The development of modern bio-medical technologies involving mammalian embryos cloning and embryonic stem cells creation and use requires new effective methods for embryos treatment. The fully-optical method of laser micromanipulation has been developed and applied for noninvasive laser nuclear transfer in preimplantation mammalian embryos. Short-pulse high repetition rate IR laser was successfully used to demonstrate enucleation, the transfer of the somatic cell under the zona pellucida of the cytoplasm, and their subsequent fusion in mouse embryos without any mechanical contact. Lifetime autofluorescence imaging of mitochondrial NADH and Raman mapping are discussed as methods for noninvasive diagnosis and analysis of embryos development.

Introduction

Today somatic cell nuclear transfer (SCNT) is the basic procedure for reproductive cloning as well as for therapeutic cloning. Recently at all steps mechanical, electrical or chemical treatments are used for SCNT, which cause side effects, and are traumatic [1].

With an increasing need in modified embryos (for therapeutic or reproduction cloning, for stem cells preparation, etc.) the development of new effective standard and stable methods to obtain and not injure viable cloned embryos becomes an urgent task.

The use of lasers for treatments and studies of biological objects has started almost at once after the implementation of the lasers and has been successfully applied with the increasing rate in many biological and medical fields [2, 3]. However, lasers application in micromanipulations with preimplantation mammalian embryos is limited today to easy opening of zona pellucida and dissection of blastocysts [4]. The expanded applications of lasers in embryology open possibilities to develop effective non-invasive methods of control, analysis and diagnostics of embryos. In this work the laser applications in embryology are discussed and the examples of successful applications are demonstrated.

Laser Embryology (Surgery and Manipulation)

The novel methods were developed using pico- and femtosecond lasers with high repetition rate for such embryo microsurgery manipulations as embryo enucleation and fusion of somatic cell with embryo. These manipulations are recently performed usually using electrofusion in special medium and have serious side effects, which are not observed at laser manipulations.

In our work, the opening of zona pellucida has been performed using a long-pulse IR laser (1.46 μm) which has become a standard tool in biology and medicine (in IVF labs). A somatic cell has been trapped with laser tweezers and inserted under zona pellucida, and pressed as close as possible to the oocyte. The somatic and oocyte cells were fused using short-pulse laser irradiation. In the future, this technique will allow effective obtaining of modified early mammalian embryos.

The unique peculiarity of laser fusion and inactivation (functional enucleation) is the possibility to fuse separate blastomeres inside a single integral embryo, without disturbing viability of surrounding organelles/neighbor cells.

Thus, preimplantation embryos can be obtained with various ploidy inside one embryo, which are able to develop to blastocyst stage. These modifications can be used both for understanding fundamental mechanisms and for practical applications.

Analyzing and Diagnostics

The methods of optical spectroscopy and imaging are developed for diagnostics of the preimplanted/treated embryos. The methods of Raman microspectroscopy and Raman mapping allow mea-

suring and localizing with high spatial resolution and high sensitivity the molecular processes in the preimplantation mammalian embryo. It has been shown that Raman spectra of embryos reveal a set of characteristic peaks. The spectra peaks positions, relative intensities as well as the intensities' spatial distribution vary for different cell types and states. That allows developing the methods of precise estimation of quality and viability of individual embryos.

The feasibility of Fluorescence Lifetime Imaging (FLIM) techniques was also used for optical characterization of the embryo states and diagnostics. The variations in fluorescence lifetimes demonstrate the difference in the metabolism of the various blastomeres of early mammalian embryos.

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STUDYING CELLULAR MECHANISMS OF FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY (FSHD) DEVELOPMENT

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Abstract. FSHD is a slowly progressive hereditary disease caused by inappropriate Dux4 gene expression in muscle cells. It was shown that FSHD affected muscles have a proinflammatory state, which may stimulate MSCs migration. Here, using a TNF α treatment as a model of inflammation in vitro and DUX4 plasmid transfection as a model of FSHD, we demonstrate the MSCs migration to inflamed and DUX4 overexpressed myoblasts. Overexpression of DUX4 increases the production and secretion of one of the major chemokines for MSCs – stromal derived factor 1 α (SDF-1 α).

Introduction

Fascioscapulohumeral dystrophy (FSHD) is one of the most commonly inherited muscular dystrophies characterized by progressive weakness and atrophy of facial, shoulder girdle, upper arm muscles. In the course of FSHD development, replacement of muscle tissue by adipose and connective tissue occurs. FSHD is genetically linked to deletion in the subtelomeric D4Z4 repeat array on chromosome 4q35 [1]. D4Z4 contractions are proposed to cause epigenetic changes, which ultimately increase expression of genes with myopathic potential, in particular *ANT1*, *FRG1*, *FRG2*, *DUX4c* and *DUX4* [2]. *DUX4* is a leading candidate gene for FSHD, and *DUX4* mRNA and protein expression was confirmed in FSHD muscles [4]. *DUX4* is a transcriptional factor that regulates the expression of genes involved in germline, early stem cell development and immune mediators. These *DUX4* target genes are aberrantly expressed in FSHD skeletal muscle but not in control muscle biopsies [3]. In vivo, mesenchymal stem cells (MSCs) appear to participate in the regenerative process of multiple tissues types as a result of various trophic mechanisms that become activated when exposed to the biochemical factors that are characteristic of an injury environment. Endogenous MSCs or a related population of endogenous cells (i.e., vascular pericytes) are likely to participate in the wound healing response by migrating to the site of tissue damage. The inflammatory mediators like interferon- γ (IFN γ) and tumor necrosis factor- α (TNF α) can regulate homing and migration of MSCs. Disturbance of muscle tissue regeneration may be one of the reasons of adipose and connective degeneration of affected muscles. It was shown the high level expression of proteins, which are associated with nonspecific response to inflammation, such as CXCL9, CXCL10, CXCL11, in the muscle biopsies of FSHD patients [5]. This proinflammatory state of FSHD muscles may stimulate MSC migration, which can differentiate to adipocytes or form a fibrosis. The aim of our issue was to investigate the influence of Dux4 overexpression on MSC migration to muscle cells and the role of stromal-derived factor (SDF-1) and CXCR4 (receptor for SDF-1) in this process.

Results

As the CXCR4-SDF-1 α axis is dominant in trafficking of MSCs, we investigated these genes expression by qRT-PCR in primary myoblasts and in muscle biopsies from healthy donors and FSHD patients. It was shown that the SDF-1 mRNA expression was increased in the course of FSHD myoblasts differentiation to compare to myoblast from healthy donors. The CXCR4 mRNA expression was increased also during myoblasts differentiation, but no differences between healthy donors and FSHD patients were observed (Fig. 1a). Analysis of CXCR4 and SDF-1 expression in muscles biopsies revealed a 3-fold increase in the SDF-1 expression (Fig. 1b). These results were confirmed by immunohistochemical assay of muscles biopsies. Inflammation in FSHD muscles was suggested by MRI studies and macrophages and T cells muscles invasion was confirmed by immunohistochemical assay [6]. In order to simulate an inflammatory environment in vitro we treated the immortal myoblasts cell line with TNF- α (20 ng/ml for 24 h). The TNF- α treatment increases SDF-1, but not CXCR4 gene expression (Fig. 2a). Using transwell migration assay (transwells with 8 μ m pore size) increased migration of adipose-derived MSCs (ADAS) to inflamed myoblasts was shown (Fig. 2b). We used *DUX4* plasmid transfection of myoblasts as a model of FSHD. Overexpression of *DUX4*, but not *DUX4c*, stimulates the migration of MSCs from adipose tissue and bone marrow (BMSC), which was blocked by pre-treatment with antio-

dies to SDF-1 and CXCR4 (Abcam). Stimulation of SDF-1 α production and secretion by myoblasts under DUX4 overexpression was revealed by ELISA (ab100637, Abcam) (Fig. 3). Thus, here we demonstrate that FSHD affected muscles had increased level of SDF-1 expression, inflamed myoblasts, like a DUX4 overexpressed myoblasts, may stimulate the MSCs migration because of increased production and secretion of SDF-1 α .

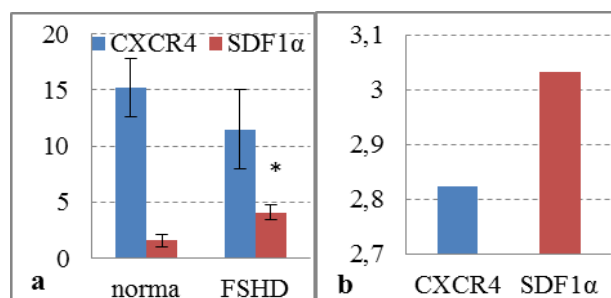


Fig. 1. Expression of SDF1 and CXCR4 genes by qRT-PCR: **(a)** the ratio of genes expression in differentiated myotubes to proliferating myoblasts from healthy donors (n = 5) and FSHD patients (n = 6) (mean $\pm$ SD; * – p<0.02); **(b)** – the ratio of genes expression in muscle biopsies from FSHD patients (n=5) to healthy donors (n=2)

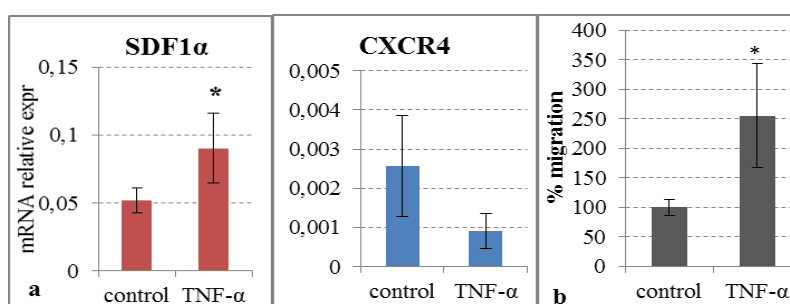


Fig. 2. TNF- α treatment (20 ng/ml) of primary myoblasts enhances SDF-1, but not CXCR4 gene expression **(a)** (n=4, * - p<0.05, Student's test) and stimulate migration of ADAS cells **(b)**

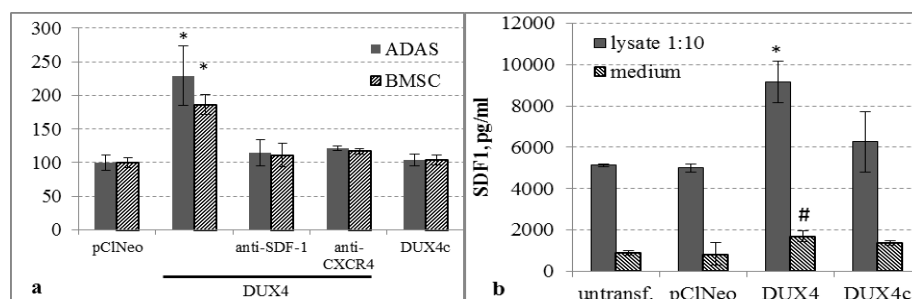


Fig. 3. Overexpression of the DUX4 gene induces the SDF-1 α production. **(a)** The overexpression of the DUX4 gene (but not DUX4c gene) in immortal myoblasts stimulates MSCs migration and this effect was attenuated by antibodies to SDF1 and CXCR4. **(b)** ELISA results for SDF1 α production by immortal myoblasts cell line. SDF1 secretion was measured in conditioned media, intracellular SDF1 α - in cell lysates (*, # - p<0.5, compare to untransfected cells)

Conclusion

Taken together our results show that MSCs can be involved in pathogenesis of FSHD due to migration at the site of FSHD affected muscles.

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STUDYING MAMMALIAN EMBRYONIC DEVELOPMENT THROUGH OPTICAL IMAGING

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Abstract. Understanding the nature and mechanism of congenital defects of different organ systems in humans has heavily relied on the analysis of the corresponding mutant phenotypes in rodent models. Optical imaging is a powerful approach to study early embryonic development in animal models. This workshop will focus on two optical imaging approaches, fluorescence microscopy and optical coherence tomography, discussing how these methods can be utilized for structural imaging of early mouse embryos in static culture, cardiodynamic and blood flow analysis, and *in utero* embryonic imaging at later stages of gestation, demonstrating how these methods can be used to assess structural and functional birth defects in mammalian models.

Studies in mouse models are invaluable for defining genetic and environmental factors affecting the anatomical and physiological development of different organ systems in humans. Since mammalian embryos develop in utero, thick layers of maternal tissue restrict embryonic imaging and hemodynamic analysis *in vivo* with high spatial and temporal resolution. To overcome this limitation, live static mouse embryo culture protocols have been developed to allow live imaging at the embryonic stages from the E6.5 to about E10.5 [1, 2]. Following these protocols, the embryos can be maintained in culture for over 24 hrs to provide an access for optical imaging while the heart is beginning to beat and remodeling into a chambered pump, blood circulation is establishing, and vasculature is forming and remodeling. Two optical approaches for live high-resolution imaging during development in cultured mouse embryos, confocal microscopy of vital fluorescent reporters and optical coherence tomography (OCT), will be discussed here.

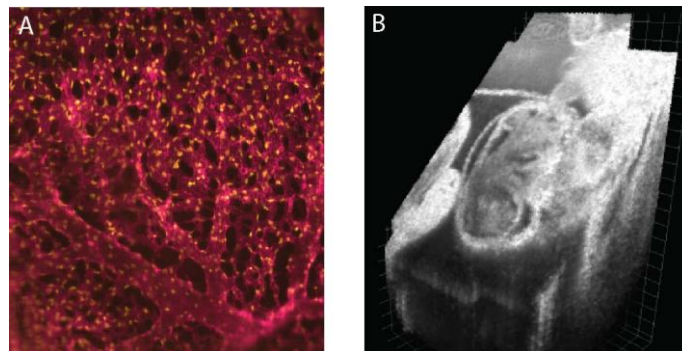


Fig. 1. Live optical imaging of embryonic tissues. (A) Confocal microscopy of yolk sac vasculature in Tg(Flk1-myr::mCherry) X Tg(Flk1-H2B::EYFP) mice. (B) OCT imaging of mouse embryonic cardiodynamics

Confocal microscopy combined with fluorescent protein reporter lines gives the advantage of performing live imaging with subcellular resolution. A number of mouse transgenic lines expressing vital fluorescent reporters have been engineered, which can be used for tracking individual cells as well as for visualization of whole developing organs. Embryonic vasculature can be visualized using two transgenic markers, Flk1-H2B::EYFP which expresses a nuclear yellow fluorescent protein (YFP) in the endothelial cells [3], and Flk1-myr::mCherry, which expresses a membrane-tethered mCherry fluorescent protein in the embryonic endothelium and endocardium [4]. Inter-crossing these markers provides a powerful combination, since the EYFP marker allows for the analysis of cell division and the tracking of each endothelial cell whereas the membrane-targeted mCherry marker reveals cell boundaries and highlights vessel organization. This combination of markers reveals the distribution of individual cells within each vessel segment (Figure 1A). A transgenic fluorescent reporter mouse model useful for hemodynamic analysis in early embryos was generated by Dyer et. al. [5]. These animals carry GFP under control of ϵ -globin promoter, which drives GFP expression in primitive erythroblasts. Blood cells brightly labeled with the GFP are first detected in the blood islands of the embryonic yolk

sac prior to the beginning of the heartbeat. A few hours after the beginning of the heartbeat (about E8.5), when the plasma flow is stronger, the blood cells leave the blood islands and join the circulation. By using this reporter in live embryo culture, these events can be directly studied.

While confocal microscopy allows for exceptional spatial resolution, its imaging depth is limited to about 200-300 μm . This limitation can be addressed by using OCT, which allows 3-D imaging millimeters into tissue, though at the expense of lower spatial resolution (2-10 μm). We have developed two approaches, which can be used for embryonic imaging with OCT. Early stage embryos (E7.5 - E10) can be imaged in culture (Figure 1B). For that, the embryos are dissected out with the yolk sac intact and cultured on the imaging stage (for up to 24 hours). We have successfully used this approach to visualize the structure of the whole embryos, analyze blood flow profiles and reconstruct cardiodynamics in 4D (3D+time) [2, 6].

This is currently the only method allowing to visualize beating heart with cellular resolution in early mouse embryos, while the whole embryo is within the imaging distance. The limitations are: (1) embryos do not survive in culture longer than 24 hours and the long-term effect of any manipulations cannot be followed, (2) embryo culture is limited to E10, since later stage embryos require placenta for survival. The second OCT embryo imaging approach is for later embryonic stages (after E12.5 till the end of gestation) *in utero* [7]. For that, the pregnant female is anesthetized, a uterine horn with embryos is exposed for imaging through a small incision in the body wall, and the embryos are imaged through the uterine wall. After the imaging, the incision is closed and the embryos continue to develop. This approach can be used for high resolution OCT imaging of different embryonic organs, such as brain, limb, and eye.

The described optical imaging approaches provide complementary analyses: while confocal microscopy in combination with vital fluorescent reporters can be used for sub-cellular analysis of vascular development and hemodynamics in the embryonic yolk sac and superficial embryonic tissues right underneath the yolk sac with sub-cellular resolution, OCT can be used for dynamic 3-D structural embryonic imaging and blood flow analysis deep within the embryonic circulatory system with resolution of single cells. These complementary approaches can be applied in a wide range of embryonic studies and can potentially answer many interesting questions about the formation of the cardiovascular system and the nature of congenital abnormalities.

Acknowledgements

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EMBRYONIC STEM CELLS CARRYING HUMAN ARTIFICIAL CHROMOSOME: PERSPECTIVES IN RESEARCH AND MEDICINE

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Abstract. Human artificial chromosomes (HACs) are a powerful DNA vector system developed recently to introduce large chromosomal fragments, genes and regulatory elements into cultured mammalian cells without affecting the host genome. This approach is devoid of known problems of viral or other vector tools such as insertional mutagenesis and unstable expression. However, the delivery of HACs directly into cells of living organism is feasible so far only via cultured cells that are first to be targeted with HACs, and then incorporated into desired tissues and organs. In this study we tried to design HAC-based approaches for combined gene and tissue-replacement therapy.

Pluripotent stem cells such as embryo-derived ES cells and autologous iPS cells seem to be an ideal choice for HAC delivery via tissue-replacement because they possess the capacity for unlimited self-renewal *ex vivo* and can differentiate into virtually any cell type of the organism both *in vivo* and *in vitro*. Here we present principal opportunity of creating embryonic stem cells carrying human artificial HAC. We successfully generated these cells via MMCT (microcell mediated chromosome transfer) procedure. After generation of the new ES cell lines carrying alphoid-tetO-HAC we checked cells for the presence of chromosomal abnormalities and artificial chromosome stability and autonomy by FISH analysis. To prove that the MMCT procedure and introduction of alphoid-tetO-HAC did not affect the stemness of ES cells, pluripotency of resulting clones was verified by immunostaining for pluripotency markers (Oct4, Nanog, SSEA1), and teratoma-formation test with further immunohistochemical analysis.

The next most interesting and important part of our experiments is the creation of a chimeric mouse using an ES cell line carrying alphoid-tetO-HAC. The creation of such mouse pursues not only practical but also a fundamental goal. It is an ability to show principal opportunity of creation an animal with completely synthetic chromosome in its genome. It is not yet known whether ES cells with synthetic alphoid-tetO-HACs can be generated and can participate in embryo development when introduced into the inner cell mass of blastocysts. Moreover, HACs have wide abilities to be used in research and medicine.

Acknowledgements

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FLUORESCENT IMAGING MODALITIES FOR MESENCHYMAL STEM CELL-TUMOR TROPISM

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Abstract. Mesenchymal stem cells (MSCs) play important roles in tumor pathogenesis. In this study we investigated various models of the interaction of tumor and stem cells (SC) using the method of confocal laser scanning microscopy (LSM). In the first model, human adipose-derived adult stem (ADAS) cells were administered into nude mice at different stages of tumor growth. In the second model, GFP (+) stem cells bone marrow was administered in irradiated mice with transplanted tumor. It was shown that SC of different origin integrate in the animal organism with transplanted tumor and can be identified by the LSM.

Introduction

It is known that MSCs play an important role in tumor pathogenesis and for this reason understanding of the interaction of stem cells and tumors is the subject of intense investigation [1]. Fluorescent methods become more common for studying SC migration in the body of recipient and investigating their involvement in tumor pathogenesis. At the same time, LSM is a standard method for detecting subcellular distribution of fluorescent endogenous and exogenous agents in vitro.

Materials and Methods

Cell culture. We used ADAS cells transfected by a gene of red fluorescent protein TurboFP635 and mesenchymal stem cells bone marrow (MSC BM) from the male GFP (+) transgenic C57/Bl6 mice.

Animals and tumors. Experiments were performed on female nude mice and male C57/Bl6 mice. Tumors HeLa Kyoto (human cervical carcinoma) and Lewis lung carcinoma were implanted in mice by subcutaneous injection of the tumor cells.

Experimental design. Two different models were studied. In the first model, TurboFP635 (+) ADAS cells were administered into the nude mice with transplanted tumor HeLa Kyoto at different stages of tumor growth (0-8 days) systemically or locally. The following groups of animals were formed: 1st group – early stage of carcinogenesis (0 day) and systemic injection of ADAS, 2nd group – early stage of carcinogenesis and local injection of ADAS, 3^d group – stage of developed tumor (8 days) and systemic injection of ADAS, control group – injection of tumor cells without SC injection. In the second model, GFP (+) MSC BM and BM cells from the male C57/Bl6 mice were administered into the sublethal irradiated (5 Gy) C57/Bl6 mice with transplanted Lewis lung carcinoma at the early stage of tumor growth (0 day) systemically. GFP (+) MSC, BM cells and tumors were injected into C57/Bl6 mice on the following day after the irradiation. The following groups of animals were formed: 1st group – irradiated mice and injection of GFP (+) MSC BM and BM cells, 2nd group – irradiated mice and injection of BM cells, 3^d group – unirradiated mice without GFP (+) MSC BM and BM cells injection.

The distributions of labeled GFP (+) MSC BM and TurboFP635 (+) ADAS cells were analyzed in the organs and tumor tissues from the 5th until 15th days after the transplantation.

Confocal laser scanning microscopy was used for monitoring TurboFP635 (+) ADAS and GFP (+) MSCs. Confocal images were captured using oil-emission lens, with magnification of 40 and 63. Fluorescent images were registered in single-photon excitation by argon laser, wavelength being 488 nm and 543 nm, power— 1-2 μ W and 12 μ W on a sample.

Results and Discussions

To obtain control spectrum of protein TurboFP635 and GFP we analyzed spectral characteristics of TurboFP635 (+) ADAS and GFP (+) MSC cells cultures expressing these proteins for further compar-

ison with tissue spectra of the organs under study. In the first model, autofluorescence in skin, muscles, heart, brain, lungs, intestine and tumor tissue of the control group of animals was found to be extremely low, while in the tissues of spleen, kidneys and liver it was bright, spectral characteristics of tissues being different from those of fluorescence-labeled ADAS. However, in the spleen of 1st, 2nd, 3^d groups a significant increase of the cells with fluorescence in the appropriate range was shown as compared to the control group. In experimental animals the areas of fluorescence consistent with the spectrum of cells with protein TurboFP635 were revealed in the tissues with low level of autofluorescence: tumors, bone marrow, and the lungs, that gave the evidence of the accumulation of labeled cells in these niches. Fluorescence of TurboFP635 (+) ADAS was found in tumor tissues and bone marrow of the 2nd group and lung tissues of the 3^d group of animals. The animals of the other groups had no fluorescence in tumor tissue, bone marrow and lung tissues, which was associated with the accumulation of TurboFP635 (+) ADAS (fig.1, A).

In the second model were analyzed the distribution of labeled GFP (+) MSC BM and their descendants in the organs and tumor tissues on the 7th, 12th and 15th days after the transplantation. The autofluorescence in the lungs, spleen, liver and the tumor tissues of the control group animals was found to be weak. In the spleen of the 1st group of animals we revealed accumulations of the cells with spectral characteristics of GFP (+) MSC on the 7th day after transplantation and by the 12 day their amount increased. However, in the liver of the 1st group of animals the same accumulations of GFP (+) cells were found only on the 12th day and by the 15th day their number decreased. The fluorescence of GFP (+) MSC was found in tumor tissues of the 1st group animals on the 12th and 15th days after transplantation. In the lung tissues we detected areas of fluorescence corresponding to the spectrum of GFP protein quite frequently encountered in the field of view on the 7th day after the injection, and scattered by the 12th and 15th days of the experiment (fig. 1, B). Our results on the distribution of labeled SC in animals-tumor carriers were confirmed by the data available in the literature on different experimental models [2].

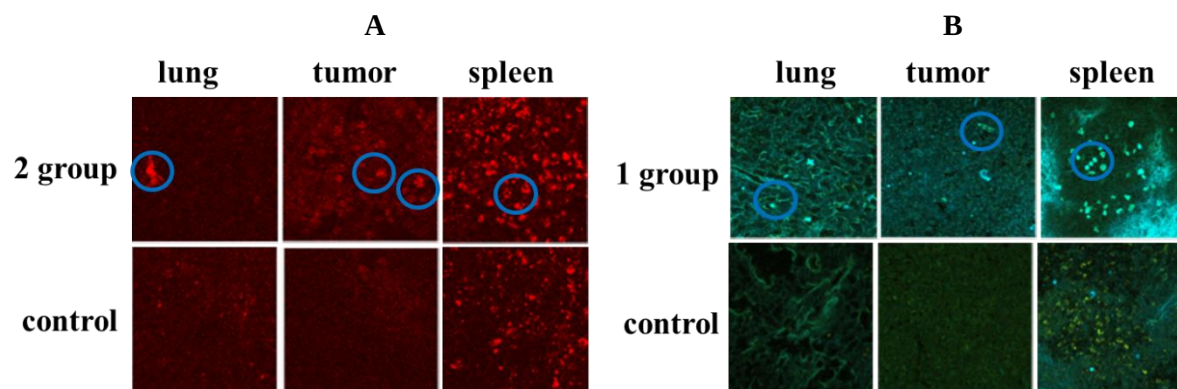


Fig. 1. Confocal laser scanning microscopy of organ tissues *ex vivo*. A – First model: fluorescence images (fluorescence excitation — 543 nm, registration — 650-710 nm, x63) of animal tissues of the 2nd and control groups; B - Second model: fluorescence images (fluorescence excitation — 488 nm, registration — 500-650 nm, x40) of animal tissues of the 1st and control groups; circle marked localization of labeled SC

Conclusion

The LSM method can be used to study the interaction of the tumor and mesenchymal SC. The cells under study — labeled ADAS and GFP (+) MSC are able to migrate in the animal organism with transplanted tumor and can be identified by the LSM.

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LASER NANOSURGERY OF PREIMPLANTATION MAMMALIAN EMBRYOS

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Abstract. Femtosecond laser light interaction with biotissue permits noninvasive surgeries in the bulk of a sample with submicrometer resolution. We briefly review the experimental background of femtosecond laser surgery. The accuracy of femtosecond laser surgery is limited by the Abbe's diffraction limit. We demonstrate new approaches to obtain the resolution of ~ 100 nm to cut the polymer or biomembrane. We summarize research applications, encompassing cell and embryos studies. We intend to use our femtosecond laser specialties in order to delve into the realm of cell signaling and gene transfection in mouse embryonic stem cells. Future trends of femtosecond laser systems and some possible applications for cell and embryos are discussed.

Introduction

The femtosecond laser has been gaining momentum in the field of laser surgery. Its high peak intensity and short pulse duration result in a light scalpel tool that negligibly heats its surroundings. In addition, advances in laser technology are making femtosecond laser systems more accessible in terms of cost and maintenance. The goal of this work is to review briefly our recent results in the laser surgery of cells and embryos.

Experimental

The optical laser setup shown in Fig. 1 includes 3 different lasers with different functions. The femtosecond Ti^{3+} : sapphire laser is used as a light scalpel, the cw Ti^{3+} : sapphire laser is used as tweezers and the pulse diode laser is used for surgery with embryos.

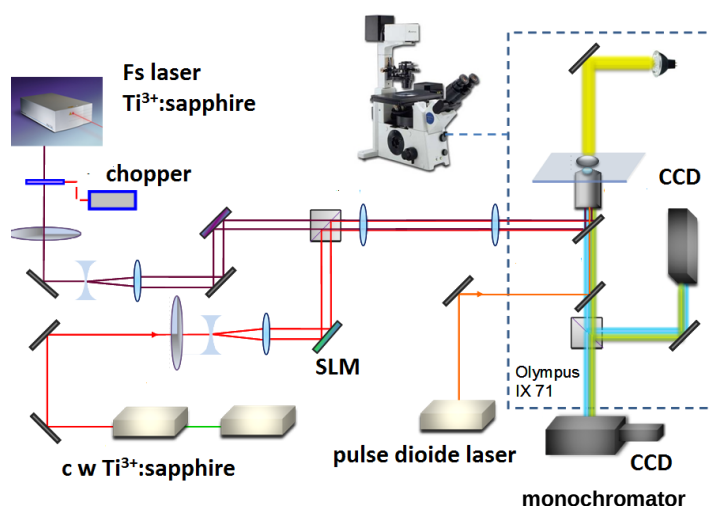


Fig. 1. The optical experimental setup

Embryos were obtained from superovulated female mice C57BL / 6 male line coupled with CBA. In our work we used original, specially designed for these experiments methods and approaches, as well as the standard techniques known in cell biology and cell engineering. Embryonic stem cells mice transformed with a vector pEF-GFP green fluorescent protein encoding were used.

Results

The resolution enhancement is achieved by using the near field approach with microlenses (dielectric spheres with a diameter $\sim 1 \mu\text{m}$). Nanoplasmonic laser surgery was used also. Figure 2 demonstrates the gold nanoparticles mediated dissection of the bacterial cell membrane. This approach uses plasmon resonances on gold nanoparticles (Au NPs) attached to the cell membrane to evoke transient and spatially defined cell membrane permeabilization.

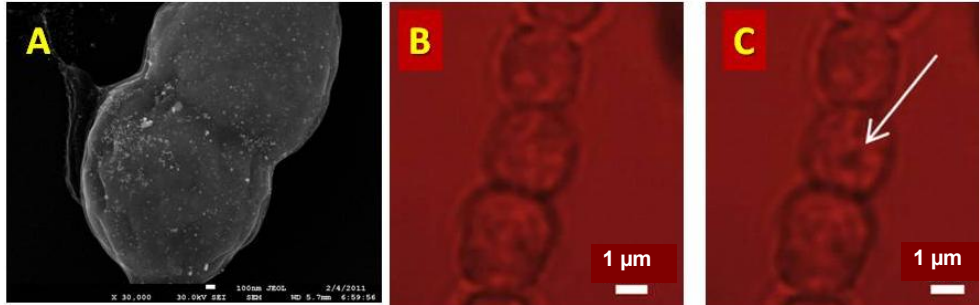


Fig. 2. Optoperforation of bacterial wall assisted by Au NPs. Excitation by femtosecond pulses. Pulse: 100 fs, 800 nm. **A**, SEM image of *Anabaenasp.* PCC 7120 covered by Au NPs; **B**, optical microscope image of *Anabaenasp.* PCC 7120 before laser treatment; **C**, optical microscope image of *Anabaena* sp. PCC 7120 after 10 ms exposition under train of laser pulses with a repetition rate of 80 MHz. Black point is the hole in the wall due to optoperforation

In the experiments with embryos, surgery embryos were isolated in step 2-blastomere which were cultured in vitro to the stage 8 cells (morula). The experiment consisted of two phases. First, zona pellucida was opened by using the diode laser so it was possible to enter the stem cell with a diameter of about 10 microns in the hole. At the second stage, the stem cells were trapped by a continuous Ti: sapphire laser 790 nm and stem cells were moved in the zona pellucida. The embryonic stem cells mice transformed with a vector pEF-GFP green fluorescent protein encoding were used in experiments. Further, the embryos were cultured in a CO₂ incubator environment M16 to the stage of blastocyst hatches (Fig. 3a, b, c, d).

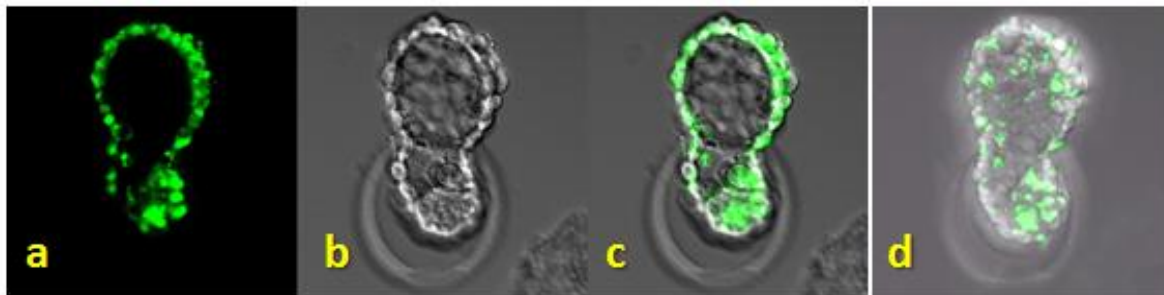


Fig. 3. a) The fluorescent embryonic stem cells are included in the trophectoderm and the internal cell. The hatched blastocyst stage is shown, b) the same blastocyst in transmitted light, c) superposition of photos a and b; and d) 3D image. Grey - 3D reconstruction

The production of chimeric mouse blastocysts by laser nanosurgery without using any other additional equipment is demonstrated in these experiments.

Acknowledgements

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APPLICATION OF FEMTOSECOND LASER SCALPEL AND OPTICAL TWEEZERS IN ASSISTED REPRODUCTION TECHNIQUES AND STEM CELL RESEARCH

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Abstract. A compact system consisting of a femtosecond (fs) laser scalpel and optical tweezers was developed. Laser-based technology of embryo biopsy (polar body as well as trophoctoderm biopsy) for pre-implantation genetic diagnosis was realized on the basis of the system mentioned. Noncontact dissection and trapping of desired amount of trophoctoderm cells was also performed successfully with the help of the system. Another intriguing application of the system lies in the field of stem cell research/gene therapy and is related to laser-based microsurgery of cell membranes for targeted delivery of extrinsic molecules into the cells and their reprogramming.

The area of ultrashort laser applications in medicine and biology has rapidly expanded over the past few decades. Due to nonlinear optical effects occurring around the focal volume of a tightly focused laser beam, utilization of femtosecond (fs) laser pulses offers several attractive advantages compared with long-pulsed or continuous wave lasers. No out-of-focus absorption and absence of significant heat or mechanical energy transfer to surrounding structures provide the highest precision and control as well as minimize the risk of adverse effects on development, growth and reproduction of living cells and organisms. Consequently, femtosecond lasers are widely used in ophthalmology for corneal surgery, in dentistry for efficient and controlled caries removal and cavity preparation, in life science for high resolution imaging of cells and tissues. One of the most promising applications of femtosecond lasers is the dissection or ablation of subcellular components [1-3] and selective permeabilization of cells, the so-called optical transfection (perforation) [4-6], enabling introduction of foreign molecules into cells. In this work several applications of fs lasers, e.g., for noncontact embryo biopsy, cell fusion and optical transfection, will be discussed.

Embryo biopsy

Embryo biopsy is one of micromanipulation and microsurgery techniques actively used for preimplantation genetic diagnosis (PGD) of monogenic diseases or chromosomal aberrations. Embryo biopsy can be carried out at different stages of embryo development. In humans, on the day of the oocyte retrieval or on the day following the fertilization one or two polar bodies (PBs) can be removed for genetic analysis. On the third day of the embryo development the so-called blastomere biopsy is performed. At the stage of blastocyst (day 5 embryo) trophoctoderm (TE) can be biopsied. All the techniques listed above have their advantages and limitations. For example, polar body removal causes no detrimental effects on the cleavage rates as PB is not involved in this process. However, genetic analysis of the PB only evaluates possible disorders of the maternal genetic component. Moreover, gender determination of the embryo is also impossible with PB biopsy. Blastomere biopsy allows determining the gender, as well as testing not only maternal but also paternal genetic material. On the other hand, there is some evidence that implantation and pregnancy rates appear to be lowered by biopsying at this stage [7]. Moreover, as blastomeres may not be identical, blastomere biopsy can lead to a misdiagnosis. It can also occur because of limited material available for analysis when polar body or blastomere biopsy is performed. To overcome this problem trophoctoderm biopsy is utilized. Multiple TE cells can be taken from the embryo to be tested, leading to a more reliable diagnosis [8].

The technique of fully noncontact embryo biopsy (without using any micromanipulators and pipettes) performed with femtosecond laser scalpel and optical tweezers is presented for the first time. Noncontact polar body biopsy as well as trophoctoderm biopsy of C57Bl/CBA mouse embryos is demonstrated. For a polar body biopsy femtosecond laser scalpel is used to dissect the outer covering of the embryo. In order to make the whole process of embryo biopsy fully-noncontact optical tweezers (Figure 1) are employed to remove the polar body out of the embryo. Trophoctoderm biopsy involves

taking cells from the outer layer of the embryo. Femtosecond laser pulses are applied to dissect 5-7 TE cells herniated through the zona pellucida. After being dissected TE cells are trapped and moved by a certain distance with optical tweezers. The biopsy efficiency and survival rates of the biopsied embryos are discussed.



Fig. 1. An example of polar body removal from 2-cell oocyte with the optical tweezers

Microsurgery of cell membranes

As a rule, in foreign literature the laser technique of delivering various compounds, such as fluorochromes, proteins, ions, and even nanoparticles, is referred to using the general term ‘optoinjection’. The term ‘optical transfection’ is used for a class of laser techniques used to introduce nucleic acids (RNA, DNA) into cells. In the majority of optoinjection cases, the Ti:Sapphire laser systems were used that generate femtosecond laser pulses at a centre wavelength of 800 nm. In the present work the injection of specified extracellular compounds into cells was implemented using the laser scalpel on the basis of an ytterbium femtosecond laser (1048 ± 2 nm, 75 MHz, ~ 115 fs) with a built-in diode pump unit. The possibility of femtosecond laser cell transfection is demonstrated by the example of introducing the plasmid DNA pEGFP-N1, encoding the green fluorescent protein (GFP), into the cells of the CHO-K1 line (Chinese hamster ovary cells). Fluorescent dyes that cannot penetrate through the membrane of living cells were used as a diagnostic tool for increasing efficacy of optoinjection by optimizing the laser radiation parameters and irradiation regimes. To determine the optimal conditions for efficient poration of the membrane and increasing its permeability, the propidium iodide dye (Sigma) was used. For the laser transfection of the cells, we used the irradiation regime and the parameters of laser radiation similar to the optimal regime and parameters found in the dye optoinjection experiment.

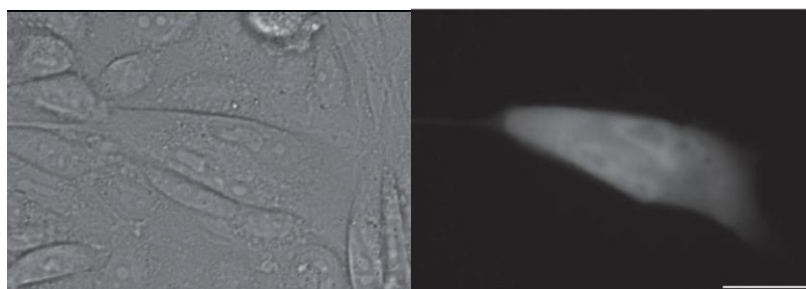


Fig. 2. Femtosecond laser transfection into the cell of the plasmid pEGFP-N1 that encodes the green fluorescent protein: (a) the CHO line cells at the next day after the laser transfection and (b) the intense fluorescence of the cell, confirming the introduction of the plasmid and the consequent expression of the GFP in the cell

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TOWARDS REAL-TIME MONITORING AND CONTROL OF STEM CELL PROCESS CONDITIONS IN MICROFABRICATED BIOREACTORS

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Abstract. Microfluidic bioreactors were pioneered over a decade ago as tools for early bioprocess development. The dramatic reduction in operating volume, the integration with optical sensors for the real-time monitoring of analytes, and the parallelization capability as a characteristic of microfluidic technology make microfluidic bioreactors an attractive tool for bioprocess development and synthetic biology. These microfabricated bioreactors have been predominantly applied for suspension cultures of microbial and mammalian cells. In this contribution, we present a microfabricated bioreactor capable of culturing embryonic stem cells in adherent culture, and monitoring the proliferation of stem cells by detecting the culture confluency.

Microfabricated bioreactors have a number of advantages that are unattainable or difficult to attain with other reactors. They include a significant reduction in use of resources (e.g. nutrients), the integration with optical detection methods for the real-time monitoring (and control) of critical process variables, an ease of sterilisation and set-up of the experiment by using disposable polymers, automation with data handling routines, and the capability to rapidly test different processing conditions. However, they have almost exclusively been applied to microbial fermentation and mammalian cell culture [1].

For this workshop, I will explain how my group at UCL Biochemical Engineering has begun to use these reactors for stem cell process development. I will explain the microfluidic design concepts we investigated and characterisations we undertook in order to arrive at a device capable of culturing embryonic stem cells under low shear and where the cells are cultured on tissue-culture polystyrene. These two features permit the culturing of cells under conditions that can potentially be linked with other culture vessels, such as existing T-flasks. Additionally, a ‘gentle’ static seeding approach further enhances comparability with existing culture methods. To obtain growth data, we developed image-processing algorithms that rapidly detect cell culture confluency, and have begun to integrate oxygen sensors to analyze bulk and peri-cellular oxygen levels. Finally, a first multiplexed platform was designed and fabricated and I will briefly touch on challenges to develop such a platform.

Acknowledgements

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Abstract. Photoacoustic (PA) imaging is a hybrid imaging modality for non-invasive detection of tissue structural and functional anomalies. The technique combines the advantageous properties of optical and ultrasound imaging. It provides the excellent contrast achieved in optical techniques with the high spatial resolution of ultrasound imaging. Some important applications of this technique include breast cancer detection, skin cancer visualization and small animal imaging. In the present work, we will discuss the design and implementation of a photoacoustic (PA) imaging system integrated into a micro-ultrasound (US) platform for co-registered PA-US imaging. In addition, we will also discuss the different applications of the respective approach especially in clinical and pre-clinical arena.

NIKON'S CELL CULTURE OBSERVATION SYSTEM: BIOSTATION CT

Catherine Kitts

Nikon Instruments Europe BV, The Netherlands

Nikon's BioStation CT is an innovated solution that combines Nikon's advanced optics and a precisely controlled environment to give an integrated system for long term live cell imaging. The BioStation CT was designed for iPS stem cell research; however, it can be used for a variety of different cell culture research. The current method of live cell imaging requires a stage top incubator and can only hold a single sample, but for a multi-user facility, acquiring images for longer than a couple of days was not possible. Therefore, Nikon developed the BioStation CT as a solution for imaging multiple samples over a couple of weeks to months. Finally, Nikon automated the entire process.

The BioStation CT has precise environmental controls for humidity, temperature, CO₂, and O₂ (optional) that can easily be adjusted. Due to the optimal environmental conditions, the health and condition of the cells are not jeopardized. It also supports a variety of different culturing formats and can hold up to 30 vessels, allowing for multiple users to use the BioStation CT simultaneously. The sample holders are machined with high precision that allows the robot to place the sample in the same position every time, leading to high reproducible results. This gives the ability to track cells over long periods of time.

The BioStation CT's microscope has the capability of five different magnifications (2X, 4X, 10X, 20X, 40X) for both phase contrast and fluorescence imaging. The phase contrast images are taken using a red colored LED in order to prevent photo-toxicity of the cells, while the fluorescence imaging option has up to five fluorescence channels to choose from. In the live imaging mode, a user can adjust the focus of the phase image, choose custom points for imaging, easily toggle between magnifications, and adjust the fluorescence exposure settings. A new feature, just released, allows for live fluorescence imaging and gives the user the option to set a fluorescence z-offset from the phase image.

Time lapse experiments are easy to setup using the user friendly interface, and each user has the option to customize their experiments. Users have the option to setup three types of observations: custom points (single FOV at different positions), full well scan, and custom tiling points (multiple FOVs tiled together to make larger, high resolution image). The interface allows you to choose the magnifications, fluorescence channels, and z-stack option for all or individual wells. In the scheduling menu, the user can set the time length and time interval they desire and then just click the start time and the scheduling menu automatically sets all the time points. Once the user has made their schedule, the BioStation CT does the rest. While the sample is being imaged over the desired time period, the user can monitor their sample images remotely using the web interface option. The web interface option also allows users to view and download new and old images to their computer. Finally, after data acquisition, Nikon offers a unique analysis software, CL-Quant. CL-Quant is a dynamic software that can analyze large amounts of data in a short period of time. Specially designed recipes allow users to apply cell count, cell proliferation, wound healing, cell motility, and iPS colony detection recipes to their data for fast analysis.

OPTICAL IMAGING TOMOGRAPHY TO STUDY DISEASE PATHOLOGY AND GENE REGULATION IN VIVO

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Abstract. Advanced optical technology enables the 3-dimensional reconstruction of luminescent and fluorescent signals for localization and absolute quantification of signals. Integrated optical/CT systems are available, but streamlined co-registration with external CT or MRI data is also possible and combines the unparalleled specificity and sensitivity of optical imaging with high-resolution structural imaging. Recent technological advances in fluorophores and optical signal detection technology have made possible rapid sequential imaging and quantitation of bioluminescent and fluorescent signals using a single instrument. This allows collecting additional data on the biological status of the tumor, such as receptor expression, invasiveness, and apoptosis rate.



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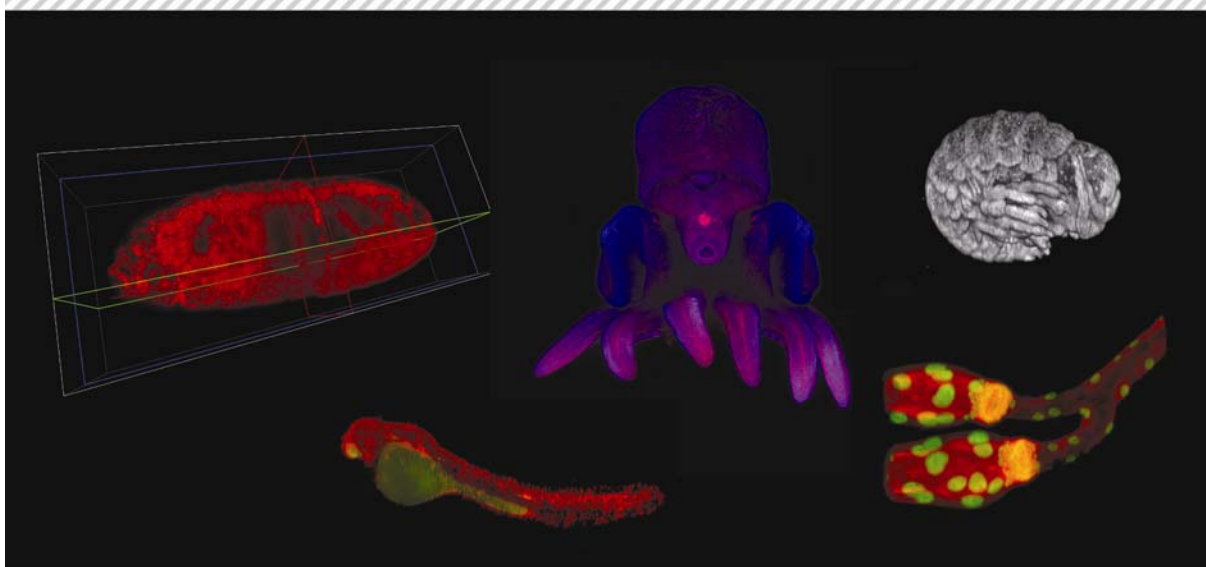


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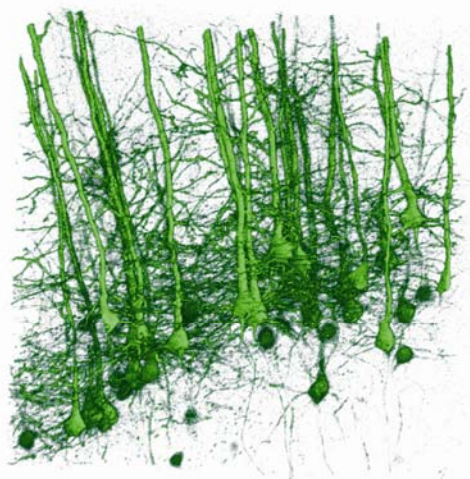


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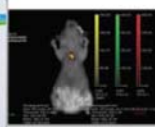
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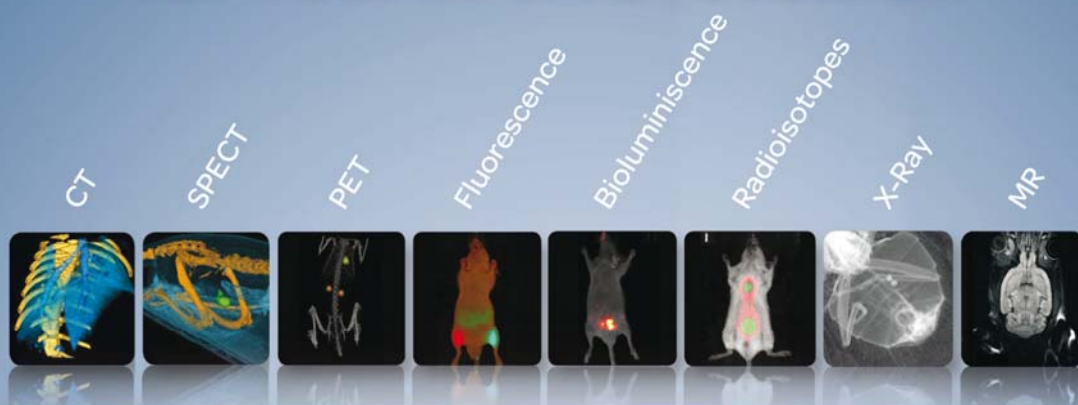


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Nikon incubation microscopy

When imaging living cells, tissues and organisms, it is essential to maintain cells in an optimal environment, which as closely as possible mimics the *in vivo* environment to obtain results that reflect real life processes. Failure to control the environment, especially during long-term time lapse studies may result in unhealthy cells that fail to divide or exhibit abnormal morphologies confounding the interpretation of the results. A constant environment also helps to define the effects of experimental variables more accurately, and provides more consistent and reliable results.



BioStation is a combined incubator and imaging system that provides a user specified automatically maintained environment for cells during culture and imaging. Potential cell stress from environmental changes and physical disturbance, when transferring culture vessels from incubator to microscope for imaging, is eliminated. With cell friendly phase and fluorescence imaging, Biostation IM-Q (for up to four concurrent studies) and BioStation CT (for multiuser/multistudies) are ideal for determining the effects of morphogens, performing protein localisation studies, monitoring the effects of gene knockout and knockdown, etc. while minimising cell stress.

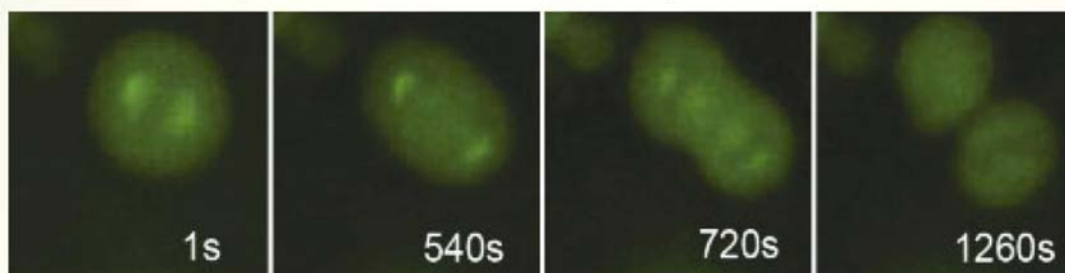
CASE STUDY: INHERITED MICROCEPHALY & BRAIN DEVELOPMENT

The inherited disease microcephaly (MCPH) is characterised by a small, but architecturally normal brain. Apart from mental retardation, there are generally no other apparent developmental abnormalities suggesting that MCPH genes may have a specific role in neurogenesis. Mutation in the ASPM gene at the MCPH5 locus is the most common cause of MCPH. Around 100 mutations have been identified but there are few genotype/phenotype correlations.

"We are trying to find out more about the function of ASPM and the effect of ASPM mutations on the developing brain," explains Dr. Jacquelyn Bond of the Section of Ophthalmology and Neuroscience, Leeds Institute for Molecular Medicine.

Using BioStation IM, Dr Bond is planning a series of rapid 4-hour live cell studies using C-terminal constructs to look at protein localisation and mitotic redistribution in the cell. "In pilot studies we could easily see the GFP-tagged protein moving to the spindle pole – it was beautiful! We are also planning to use confocal imaging, especially FRET and FRAP, for protein localisation, protein interaction and trafficking studies using our Nikon inverted microscope configured for TIRF and four-laser confocal imaging. By inserting a GFP- or RFP-linked ASPM fragment construct into cells, we can photobleach the spindle pole and determine, by the reappearance of fluorescence, whether further ASPM-GFP fusion proteins migrate into the area, indicating a transitory interaction with the spindle".

"There is still a great deal to find out about MCPH," Dr Bond comments.



"We have affected families that cannot be mapped to known loci indicating that there are likely to be further MCPH genes. We also need to find out more about factors that regulate the transcription and activity of MCPH genes. Besides being of benefit to families affected by MCPH, research contributes to the understanding of brain development in general, which may have an impact on other neurodevelopmental diseases."

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