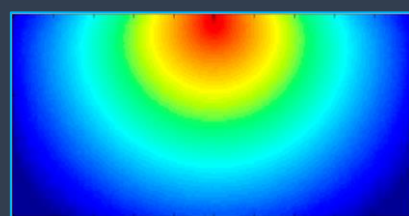
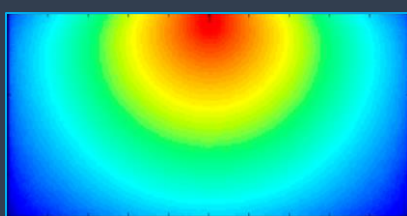
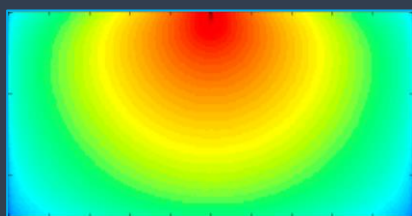
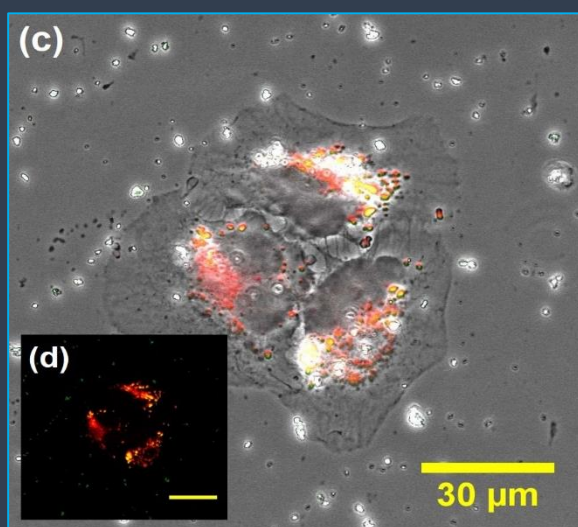
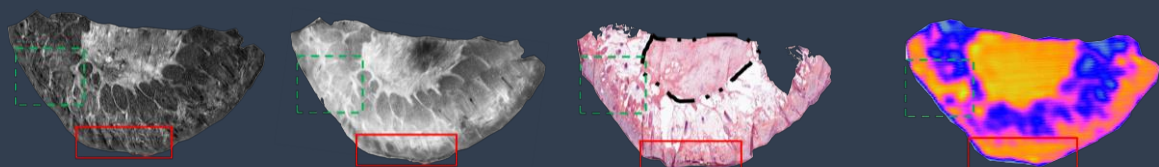


Special Issue

"Years in Biophotonics: 70th Anniversary of Prof. A.V. Priezzhev"

Journal of Biomedical Photonics & Engineering



Special Issue Editors

**Mikhail Yu. Kirillin, Juergen Lademann,
Valery P. Zakharov, and Valery V. Tuchin**

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Postal address:

39B Lukacheva st., room #301

Samara 443086, Russian Federation

Special Issue

“Years in Biophotonics: 70th Anniversary of Prof. A.V. Priezzhev”

Special Issue Editors

Mikhail Yu. Kirillin, Institute of Applied Physics RAS, Nizhny Novgorod, Russia

Juergen Lademann, Charité – Universitätsmedizin Berlin, Germany

Valery P. Zakharov, Samara National Research University, Russia

Valery V. Tuchin, N.G. Chernyshevsky Saratov National Research State University,
Institute of Precision Mechanics and Control of the RAS, Saratov,
Samara National Research University, Samara, Russia

Used Figures from this issue on the Cover

Anna N. Yaroslavsky, et al. Delineating nonmelanoma skin cancer margins using terahertz and optical imaging, 010301, Fig. 3 (reformatted, adopted).

Daria A. Loginova, et al. Probing depth in diffuse optical spectroscopy and structured illumination imaging: a Monte Carlo study, 010303, Fig. 1 (reformatted, adopted).

Y.-C. Lin, et al. Fluorescence analysis of nanodiamond-berberine complex interaction with living cells for nanoparticle mediated drug delivery, 010305, Fig. 6 (cutted).

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Foreword to the Special Issue Years in Biophotonics: 70th Anniversary of Prof. A.V. Priezzhev

We are pleased to present the special issue of JBPE dedicated to the celebration of the 70th anniversary of Prof. Alexander V. Priezzhev and aimed to honor his contribution to the field of biophotonics.



Alexander V. Priezzhev was born on April, 3, 1947. He graduated and received his PhD degree from the Department of Physics, M.V. Lomonosov Moscow State University (Moscow, Russia) in 1971 and 1975, respectively. Currently he is the head of the Laboratory of Laser Biomedical Photonics at M.V. Lomonosov Moscow State University. As a principal investigator or research fellow he was involved in numerous national and international research projects in the area of medical physics and biomedical optics; he co-authored more than 350 peer-reviewed papers, books and conference proceedings. Alexander V. Priezzhev has been awarded by the Ministry of Healthcare of Russian Federation for long lasting activities in development of novel optical techniques for medical applications. He also received the award by the Ministry of

Science and Education of Russian Federation for achievements in research and education activities. His areas of expertise cover biophysics, biophotonics, biomedical optics, laser medicine, medical physics, physics of biological fluids, nanobiophotonics, and optical and laser measurement techniques.

This issue presents a collection of state-of-the-art research papers in the field of biophotonics closely related to the scientific interests of Prof. A.V. Priezzhev

The paper by [Anna N. Yaroslavsky](#) and co-authors from University of Massachusetts in Lowell and Massachusetts General Hospital (USA) reports on multimodal terahertz-optical imaging for delineating nonmelanoma skin cancers providing high increase in sensitivity and specificity of cancer detection and demonstrating great potential for intraoperative assessment of tumor margins.

[Alexander Krasnovsky](#) and [Anton Kozlov](#) from A.N. Bach Institute of Biochemistry RAS and M.V. Lomonosov Moscow State University (Russia) discuss photooxygenation rates of singlet oxygen traps upon direct laser excitation of oxygen in air-saturated organic solvents and water. The results are important for both analyses of oxygen photonics and dosimetry of laser radiation in biomedical experiments.

Diffuse optical spectroscopy is one of the perspective diagnostics techniques for a variety of biomedical applications connected with monitoring of tissue oxygenation. The Monte-Carlo simulation of signal formation in such system is performed by [Daria A. Loginova](#) with co-authors from the Institute of Applied Physics RAS and N. I. Lobachevsky State University of Nizhny Novgorod (Russia). The main aim of this paper is to evaluate the probing depth in different probing configuration that cannot be measured directly, however, it is very important for the optimal technique performance. The authors study different illumination patterns and their efficiency for diffuse optical spectroscopy applications.

Investigation of different algorithms of tissue structural analysis based on optical coherence tomography is presented in paper of [Dmitry S. Raupov](#) with co-authors from Samara National Research University (Russia). The authors propose anisotropic diffusion speckle noise reduction

pre-processing and multimodal texture approach for identification of skin cancer in optical coherence tomography images, involving Haralick texture features, fractal dimension, complex directional field features and Markov random field method. High efficiency of multimodal texture analysis in differentiation between tumor and healthy tissue is demonstrated.

[Yu-Chung Lin](#) with co-authors from National Dong Hwa University (Taiwan), Institute of Physics (Taiwan) and P. N. Lebedev Physics Institute RAS (Russia) reports on the perspectives of functionalized nanodiamond complexes in biomedical applications. In particular, nanodiamond-berberine complexes are demonstrated to be an efficient tool where nanodiamonds serve as a drug carrier for target delivery while berberine acts as an antimicrobial drug. Fluorescence imaging techniques are employed for delivery monitoring.

[Elina Genina](#) with co-authors from Saratov National Research State University, Institute of Precision Mechanics and Control RAS and National Research Tomsk State University (Russia) reports specific features of the optical clearing of the human dura mater in vitro under the action of glucose solutions. The relative diffusion coefficients of glucose are calculated based on the analysis of the collimated transmittance kinetics. The possibility of efficient reduction of light scattering is demonstrated.

[Sergey N. Savenkov](#) with co-authors from T. Shevchenko National University of Kyiv (Ukraine), M.V. Lomonosov Moscow State University (Russia), and University of Eastern Finland (Finland) reports on application of optical diagnostics for material characterization. The authors employed Mueller matrix polarimetry to study the samples of microcrystalline cellulose with different porosity. It was demonstrated that this approach provides high accuracy in non-destructive porosity measurements.

[Inga Saknite](#) with co-authors from University of Latvia and Riga Stradins University (Latvia) report on application of optical diagnostics for monitoring short-term and long-term effect of novel moisturizing cream on human skin. Combined employment of optical spectroscopy and skin impedance measurements allowed them to monitor skin hydration at different measurement depths.

[Herbert Schneckenburger](#) and co-authors from Aalen University (Germany) demonstrate high potential of modern microscopy modalities for biomedical studies at cellular level. Different focus positions with respect to the sample are discussed. The considered microscopy modalities include light scattering microscopy with angular resolution and structured illumination microscopy.

[Olga Baum](#) and co-authors from Institute of Photonic Technologies (Russia), B.I. Stepanov Institute of Physics (Belarus), State Research Institute of Eye Diseases (Russia), and M.V. Lomonosov Moscow State University (Russia) presents the concept of the laser activation in drainage system function of eye sclera for two anatomic channels of eye fluid outflow. The study combines experiment with extensive theoretical modelling that allowed to calculate thermal stresses within eye tissues and predict development of cavitation processes in eye sclera that is key for optimizing modes of laser glaucoma treatment.

[Leonid Bass](#) with co-authors from Institute of Applied Mathematics RAS (Russia), University of Oulu (Finland) and Institute of Applied Physics RAS (Russia) reports on finite difference approach to solution of the radiative transfer equation (RTE) in general form both for stationary and non-stationary cases. Obtained solutions are compared with the results of Monte Carlo simulations and solution of RTE in diffusion approximation. Applications of the finite difference approach to blood sensing and vessel imaging are reviewed.

In overall, the papers collected in this special issue demonstrate well the exciting potential of biophotonics for biomedical studies and applications, their wide spread throughout the world.

On behalf of all authors, we would like to congratulate Alexander Vasilyevich on his 70th birthday, and wish him many health, many new ideas and discoveries, and successes in all kinds of activities!

Special issue Editors:

Mikhail Yu. Kirillin, PhD, DSc (Tech)

Laboratory of Biophotonics,
Institute of Applied Physics RAS,
Nizhny Novgorod, Russia

Juergen Lademann, Prof. Dr. Dr.-Ing.

Center of Experimental and Applied Cutaneous Physiology,
Department of Dermatology, Venerology and Allergology,
Charité – Universitätsmedizin Berlin, Germany

Valery P. Zakharov, PhD, DSc

Department of Laser and Biotechnical Systems,
Samara National Research University,
Samara, Russia

Valery V. Tuchin, PhD, DSc

Research-Educational Institute of Optics and Biophotonics,
Saratov National Research State University,
Institute of Precision Mechanics and Control of the RAS,
Saratov, Russia
Samara National Research University, Samara, Russia

To the Jubilee of Professor Alexander Priezzhev

On April 3, 2017 Professor Alexander Priezzhev, the Head of the Laboratory of Laser Biomedical Photonics of the Physics Department and International Laser Centre of M.V. Lomonosov Moscow State University (MSU), a recognized expert in biophysics, actively working in the field of biophotonics, biomedical optics, and nanobiophotonics, celebrated his jubilee 70-th birthday.

Alexander Priezzhev was born in the family of a specialist in hydraulic work building, well-known in the USSR, who worked in the Glavsevmorput' (Northern Sea Route Headquarters) system for many years. His activities included the construction of ports at the Arctic Ocean shore (Igarka, Dudinka, etc.) and the work in Vietnam.

As early as studying at school, Alexander was fascinated by physics. He entered the evening school at the MSU, where the lectures were delivered by Russian outstanding physicists, B.B. Bukhovtsev, V.D. Krivchenkov, V.G. Zubov, and M.A. Leontovich. When he was a second-year University student, he began research work at the chair, headed by Prof. Sergey P. Strelkov, which at that time had an unofficial name of cybernetics chair. After the third year of study, he began working with Victor I. Shmalgausen who was a supervisor of his graduate work and postgraduate study. The graduate work was devoted to the solution of fundamental and applied problems of optimal control. The research in this field was continued in the postgraduate study, and in 1975 Alexander defended his PhD thesis. After the PhD defence, under the general supervision of Prof. Sergey A. Akhmanov – new head of the chair, Alexander carried out a substantial cycle of research work on mathematical modelling and experimental studies of intracellular hydrodynamics in cooperation with Prof. Yuri M. Romanovsky, the head of the Mathematical Biophysics group, and the colleagues from the Institute of Theoretical and Experimental Biophysics of RAS in Pushchino. It is worth noting that his studies on laser Doppler anemometry in application to biophysics were largely affected by the communication with Bronyus S. Rinkevichyus, presently a professor in the National Research University "MPEI", one of the pioneers of the method in Russia, with whom Alexander continues to collaborate up to now. The first results of laser measurements of the protoplasm motion velocity in living cells were published in 1978 in the "Biophysics" and "Quantum Electronics" journals. Later not only the self-oscillatory but also the autowave motions in cells were studied theoretically and experimentally. In particular, the existence of a standing-wave mode in the autowave process of amoeboid motility of *Physarum mixomycete* plasmodium was found. These results corresponded to the international level, and, in particular, drew the attention of Professor Karl-Ernst Wohlfart-Botterman from the University of Bonn, a distinguished expert in cell biophysics.

In 1988, the first laser Doppler microscope for the examination of non-muscular biological motility was developed and rapidly published in the MSU Department of Physics Preprint. This microscope was displayed at the exhibition of scientific developments of the Council for Mutual Economic Assistance and awarded an honorary diploma.

In 1989-1990 on the suggestion of Sergey A. Akhmanov and in collaboration with Vyacheslav M. Gordienko, Alexander began working on the construction of Doppler lidars for remote measurement of wind velocity in the atmosphere. The lidars based on the solid-state Nd:YAG laser, the continuous-wave CO₂ laser and the pulsed TEA-CO₂ laser were made, using which he

performed atmospheric wind velocity measurements from the high-rise meteorological tower in the town of Obninsk and from the marine platform in the Black Sea.

Yuri M. Romanovsky and Alexander have developed and starting from 1980 for many years delivered a new lecture course “Laser and computer experiment in modern biophysics.” In 1982 Yuri L. Klimontovich, an outstanding expert in chaos and turbulence theory and the author of well-known books on synergetics, invited Alexander to develop and teach jointly the course “Turbulence” completed with the experimental methods of the turbulence studies.

The most fruitful research and science-organising activity of Alexander began in the late 1980s - early 1990s and was related to the solution of problems in the field of laser biomedical diagnostics and the initiation and development of active international cooperation. As early as 1986 initiated by Sergey A. Akhmanov, the first international conference on Lasers Application in Life Sciences (LALS) in Prague, Czechoslovakia was held, and Alexander was one of its major organisers. All subsequent LALS conferences were held in more than ten countries under his active participation as one of the key organisers. A few last conferences were initiated by him and successfully held due to his great energy and efforts, namely, the conferences in Taipei (2008), Oulu (2010), Ulm (2014), and Shenzhen (2016), largely due to his fruitful international links.

In these years, the in-depth studies carried out by Professor Priezzhev in the fields of optics and biophysics of blood, its rheological properties, the interaction of blood cells with laser radiation, the fundamental problems of elastic and quasielastic scattering of light by biological cells and tissues made him one of the world leaders in biomedical optics. In the same years, the fruitful collaboration with Saratov State University (SSU) began. He actively took part in the implementation of large-scale research, education and publishing projects together with the Chair of Optics, later renamed as the Chair of Optics and Biophotonics of SSU. It would be adequate to mention the book by A.V. Priezzhev, V.V. Tuchin and L.P. Shubochkin [1], which was the first monograph in the USSR and one of the first ones in the world, as well as the first monograph in the world Handbook of Optical Biomedical Diagnostics (2002). The latter has become a reference book in many laboratories of the world, recently its two-volume second edition [2] was issued, and earlier it was published as a two-volume collective textbook in Russian [3,4].

Alexander is a brilliant lecturer and successful scientist, regularly invited for delivering lectures in the leading world centres on optical biophysics and for taking part in the programme and organising committees of numerous leading international conferences. During the period from 1983 to 2017 he was awarded nearly 70 scientific and educational grants for carrying out research and delivering lectures in the leading world universities.

Alexander is a member of SPIE-the International Society for Optics and Photonics. From the very beginning of SPIE activity in Russia he directed much of his efforts to international activity within the frameworks of this society. He is one of the founders of world-wide known SPIE symposium on biomedical optics BiOS in the USA and its European analogue EurBiOS. Thanks to Alexander's activity and help up to ca. 100 Russian scientists took part in the European symposium annually.

In the same years, starting from 1996 to the present day Alexander was the soul and ideological inspirer of the annual international school and conference on optics, laser physics, and biophotonics (Saratov Fall Meeting) <http://sfm.eventry.org/2016/>.



Saratov Fall Meeting-2016

It is difficult to list all conferences, in the organisation of which Alexander took part. We would like to mention only one more of them, the annual international conference on Advanced Laser Technologies (ALT), where for many years he is responsible for biophotonics topic. In particular, quite recently, in September 2016, a successful conference ALT'16 comprising a large number of interesting talks was held in Galway (Ireland), and the next one is currently being prepared to be held in Pusan (Republic of Korea) in September 2017. At present the preparation of the special issue of Journal Biomedical Optics, devoted to the conference ALT-2016, is about to be finished [5].

The high activity and responsibility of Professor Priezzhev in training world-class young specialists and his ability to organise perfect and mutually beneficial international cooperation is worth special attention. We present only three examples here. The first example is the long-lasting collaboration with Professor Juergen Lademann from the Charité University Clinic of Humboldt University (Germany). The second remarkable example is the fruitful cooperation during 15 years with Professor Risto Myllylä from the University of Oulu (Finland), where two of his former students are working now. One of the important results of the mutual research is the well-known monograph [6]. And the third example is the successful and also long-time collaboration with Professor Chia-Liang Cheng from the National Dong Hwa University, Hualien, Taiwan.

The research and science-organising activity of Alexander is highly appreciated in Russia and abroad. For example, from 1997 he fills the position of Adjunct-Professor at School of Biomedical Engineering, Science, and Health Systems of the Drexel University (USA). On the eve of the jubilee, Alexander was awarded the MSU First Prize for the scientific publications in the frames of the MSU Programme of Development (2016). This award characterises well the scientific

achievements, wide scope and prospects of the scientific field, developed by Alexander at present time, it is sufficient just to read the titles of some papers for which this prize was awarded [7-18].

The total number of 19 PhD theses were successfully defended under the supervision of Professor Priezzhev. The main field of study in these works was biomedicine and biophotonics. Under his supervision and with his participation multiple national and international research projects in medical physics and biomedical optics were carried out, he published more than 350 papers in refereed journals, books and conference proceedings.

Alexander pays much attention to the important work in the Russian and international journals as a member of editorial boards, invited editor or referee, e.g., in such journals as *Quantum Electronics* (from 2000), *Biomedical Optics* (1997–2010); *Journal of Laser Medicine* (from 1997), *Journal of Biomedical Photonics & Engineering (Russia)* (from 2014), *Journal of Physics D: Applied Physics* (2005) and many others.

We congratulate our dear friend and colleague in mutual research and science-organising projects Alexander Priezzhev with honourable 70th anniversary and wish him new interesting projects, good students and strong health.

Yuri M. Romanovsky,
M.V. Lomonosov Moscow State University

Valery V. Tuchin,
Saratov National Research State University

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Delineating nonmelanoma skin cancer margins using terahertz and optical imaging

Anna N. Yaroslavsky^{1,3*}, Cecil Joseph¹, Rakesh Patel¹, Alona Muzikansky², Victor A. Neel³, and Robert H. Giles¹

¹ Department of Physics and Applied Physics, University of Massachusetts, Lowell, 1 University Ave., Lowell, MA 01854

² MGH Biostatistics Center, Massachusetts General Hospital, 50 Staniford Street, Boston, MA 02114

³ Department of Dermatology, Massachusetts General Hospital, 50 Staniford Street, Boston, MA 02114

* e-mail: Anna_Yaroslavsky@uml.edu

Abstract. We evaluated terahertz reflectance imaging and a combination of terahertz and optical techniques for delineating nonmelanoma skin cancers. After imaging, each terahertz pixel of each specimen was classified as cancerous or normal using a previously determined threshold. Subsequently, cancerous terahertz pixels were re-evaluated with polarization-sensitive optical technique. This additional information enabled us to re-classify each cancerous terahertz pixel using morphological appearance of the structures in the optical images. If the structures in the terahertz pixel appeared normal, the pixel was considered normal. The generalized linear mixture model was implemented to determine the sensitivity and specificity of each method. The model tested the probability of each terahertz pixel being diagnosed as a match with histology. For terahertz imaging alone, the sensitivity and specificity were 82% and 94%, respectively. For multimodal imaging, the sensitivity and specificity increased to 96 % and 99%, respectively. Multimodal terahertz-optical imaging has potential for the intraoperative assessment of tumor margins. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: imaging, cancer.

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1 Introduction

More people are diagnosed with nonmelanoma skin cancer (NMSC) in the United States yearly than with all other cancers combined. Most of these patients undergo traditional excisional surgery, which is performed without objective intraoperative tumor margin evaluation. Standard surgery uses the visual size and differentiation of the lesion to determine surgical margins. Resected specimens are forwarded to pathology, which examines only up to 2% of the surgical margin. A minority of the patients (~25%) are treated using Mohs Micrographic Surgery (MMS), which uses histopathological frozen sections and evaluates 100% of the margins during the surgical procedure. The MMS surgery continues till the margins are clear of cancer. MMS achieves up to 99% cure rates and significantly limits the morbidity compared to non-Mohs procedures. However, MMS is not available at all dermatology facilities. Also, it is not considered appropriate for treating 75% of non-melanoma skin cancers. Thus, several techniques are currently under investigation for intraoperative demarcation of cancer margins. These techniques include confocal microscopy [1, 2], optical coherence tomography (OCT) [3, 4], endogenous and exogenous polarization and fluorescence anisotropy imaging [5-9], and Raman Spectroscopy [10, 11]. Terahertz pulsed imaging has also been investigated for skin cancer treatment [12-15].

Recently, we have proposed a combination of continuous-wave terahertz imaging (CWT) and polarization sensitive reflectance optical imaging (PLI) as a multimodal approach to intraoperative delineation of nonmelanoma skin cancers [15, 16]. The primary reason for interest in cancer imaging at terahertz frequencies is intrinsic contrast. However, due to the relatively long wavelengths, spatial resolution of terahertz frequencies is insufficient for visualization of tissue morphology. Conversely, optical imaging enables higher resolution but often lacks contrast for rapid and reliable tumor delineation. Therefore, in this

contribution we have experimentally tested and statistically evaluated a multi-modal CWT and PLI approach for nonmelanoma skin cancer demarcation. We imaged ten fresh specimens of NMSC using both the PLI and CWT imaging systems. We utilized terahertz data obtained in our previous study [16] to establish a threshold for cross-polarized terahertz reflectance signals between normal and tumor tissue. Using the established threshold we delineated tumor margins in CWT images acquired in this study. Then we re-evaluated regions outlined as cancerous in CWT using PLI. Finally, we statistically evaluated and compared the imaging results yielded by CWT and a combination of CWT and PLI using H&E histology as a gold standard.

2 Materials and Methods

2.1 Tissue Handling

Fresh discarded tissue specimens were obtained from the Massachusetts General Hospital within 40 minutes after surgeries under an IRB approved protocol. In total, seven squamous carcinomas and three basal cell carcinomas were imaged for this study. The samples came from 7 male and 3 female patients following Mohs micrographic surgery. The patient ages ranged from 53 to 77. The specimen sizes varied approximately between 6 mm and 20 mm in the lateral dimensions and from 3 mm to 7 mm in depth. The respective data are summarized in Table 1. All cancer specimens included tumor and normal regions. Upon arrival to the Advanced Biophotonics Laboratory, the samples were quickly mounted in an aluminum sample holder with a quartz window (z-cut quartz for THz and amorphous quartz for optical). Mounted specimens were imaged with the optical system and terahertz systems. The dermal side of the tissue was imaged and the sample was backed by gauze soaked in phosphate buffered saline (PBS) to avoid dehydration during the

Table 1 Nonmelanoma Skin Cancer Specimens.^a

Sample #	Gender	Age	Diagnosis	Size (mm)
1	F	74	SCC	22 x 13 x 5
2	F	74	SCC	23 x 12 x 6
3	M	56	SCC	13 x 13 x 4
4	M	71	BCC	9 x 4 x 3
5	M	73	SCC	22 x 13 x 7
6	M	73	SCC	22 x 12 x 5
7	M	66	SCC	17 x 15 x 4
8	F	74	BCC	20 x 11 x 5
9	M	77	SCC	18 x 17 x 3
10	M	53	BCC	16 x 12 x 3

^a BCC – Basal Cell Carcinoma, SCC – Squamous Cell Carcinoma

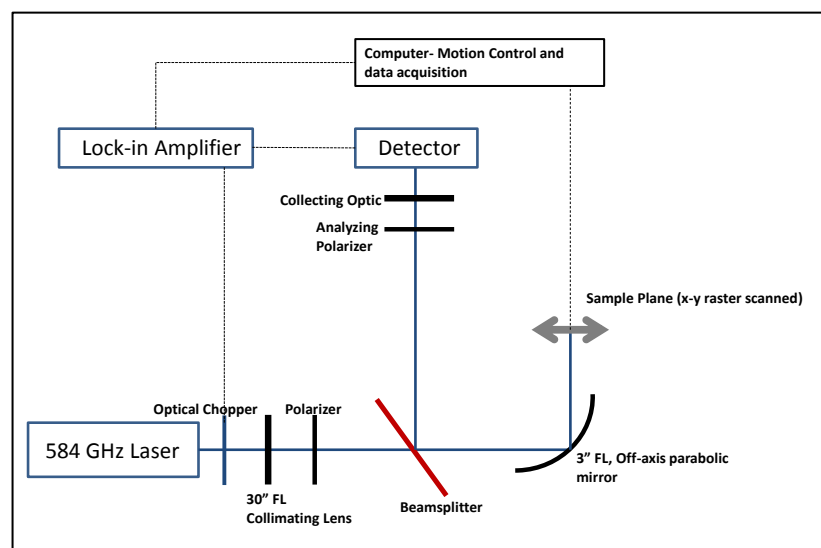


Fig. 1 Terahertz Imaging System.

measurements. Following imaging, the specimens were processed for frozen H&E histopathology from approximately the same *en face* planes that were imaged.

2.2 Terahertz imaging

The CW Terahertz system used for this experiment has been described in detail previously [16]. A lay-out is presented in Figure 1. A custom designed and built CO₂ optically pumped far-infrared (FIR) gas laser was used as the source. The system operational frequency was 584 GHz (513 μ m). The vertically polarized beam had an output power of 10 mW. For detecting the reflected signal we used a liquid Helium cooled silicon bolometer manufactured by IR Labs (Tucson, AZ). The detector's noise equivalent power (NEP) was 1.13×10^{-13} W/ $\sqrt{\text{Hz}}$, responsivity was 2.75×10^5 V/W, response time was 5 ms and gain was 200. System optics focused the beam down onto the imaging plane and the samples were

raster scanned across the beam spot using an automated x-y stage. The beam full width at half max (FWHM) at the focus was measured to be 0.67 mm. The normally incident reflectance signal was registered. Prior to entering the detector, the signal passed through an analyzing wire-grid polarizer. For these experiments, the analyzer was set to collect the cross-polarized remittance. An optical chopper was used to modulate the THz beam and a lock-in-amplifier was used to collect data. The dwell time per point in each image was about 150 ms and the system's signal-to-noise ratio (SNR) was measured to be 65 dB. The motion control and data acquisition was synchronized using a LabView® program.

After acquisition, the images were calibrated against the full-scale return of a flat front surface gold mirror. Cross-polarized terahertz reflectance image data was normalized using the formula:

$$R_{THz} = \frac{I_{Meas}}{I_{full\ scale}} \times 100\ %,$$

where R_{THz} is the normalized terahertz cross-polarized reflectivity value, I_{Meas} was the calibrated reflectivity of the pixel, and $I_{full\ scale}$ is the full-scale return from a flat front-surface gold mirror.

We determined a threshold reflectivity for cancer using the data from our previous study [16], where we measured the mean normalized terahertz reflectivity values for tumor and non-tumor structures by comparison with frozen H&E stained histopathology. Normalized terahertz reflectivity value of 0.17, which corresponds to the mean tumor normalized reflectivity plus one standard deviation, was selected as a threshold, i.e., each terahertz pixel (0.5 mm x 0.5 mm) was classified as normal if its normalized reflectivity was greater than 0.17, and as tumor if its normalized reflectivity was less than 0.17. Based on this classification, acquired terahertz images were converted to binary black and white maps of tumor and normal tissue.

2.3 Optical Imaging

An optical imaging device is shown in Figure 2. A xenon arc lamp (Lambda LS, Sutter, Novato, CA) combined with a narrow bandpass filter centered at 440 nm, with full width at half maximum of 10 nm, was used as the illuminator. The samples were illuminated at the oblique angle of approximately 50° from the sample plane. The geometry was optimized to provide uniform illumination of the imaged field. Images were acquired using a CCD camera (CoolSnap Monochrome Photometrics, Roper Scientific, Tucson, AZ) with an attached 0.5X Rodenstock lens. To enable polarization imaging, linearly polarizing filters (Meadowlark Optics, Frederick, CO) were placed in pathways of the light incident on the sample and light collected by the camera. Reflectance co- and cross-polarized images were acquired with an analyzing polarizer oriented parallel and perpendicular to the polarization of the incident light, respectively. The system's field of view was 2.8 cm x 2.5 cm, and a lateral resolution was approximately 15 μm. Optical wide-field images were acquired in less than a half a second.

Optical polarization images were processed using the following formula:

$$PLI = I_{co} - G * I_{cross},$$

where PLI is the polarized light image, I_{co} is the co-polarized image, I_{cross} is the cross-polarized image, and G is a calibration factor (0.99 for the presented system) which accounts for the bias in the system towards either polarization. The image acquisition and processing were performed utilizing Metamorph (Molecular Devices LLC., Sunnyvale, CA).

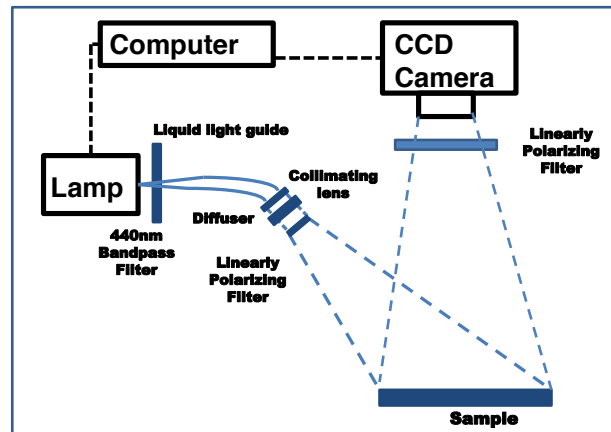


Fig. 2 Optical Imaging System.

2.4 Histopathology

En face frozen H&E histopathology was processed from the tissue samples. After imaging, several 5 μm thick slices were cut from the imaged specimens at the approximate planes that were imaged. Then the tissue sections were transferred to glass slides and stained with hematoxylin and eosin (H&E). Histopathology slides were digitized using a Zeiss Axioscope microscope (Zeiss, Germany, 5X objective lens, NA 0.13). The terahertz and optical images were compared with corresponding digitized histopathology.

2.5 Data Analysis and Statistics

For comparative evaluation of the results yielded by terahertz versus combined terahertz and optical imaging we overlaid the terahertz, optical, and histopathological images [8]. To assess the sensitivity and specificity of cross-polarized continuous-wave terahertz imaging and to quantify the enhancement to sensitivity and specificity provided by combining optical polarized light imaging with terahertz imaging we devised statistical analysis to overcome the limited number of specimens that were available for the project. We have constructed generalized linear mixture model. This model treats the tumor specimens as repeat experiments. Each terahertz pixel of each specimen (lateral size of 0.5 mm × 0.5 mm) was considered as an individual statistical data point. Then we determined the probability of each terahertz pixel being diagnosed as a match with histology as a function of the method (terahertz or combined optical/terahertz). For sensitivity tests, only the pixels, which were defined as cancer in histopathology were employed, whereas for specificity, only the pixels that were defined as normal were used. The number of statistical data points varied between specimens and ranged from 8 to 474 (as presented in table 2). We derived a sensitivity and specificity score for each specimen using repeated observation model with correlation structure taking into account the inherit correlation of adjacent statistical data points. We also determined the overall global scores across all 10 specimens. In addition, we calculated a p-value for the

difference between the terahertz and combined terahertz-optical methods per each sample using McNemar's test.

3 Results and Discussion

Example images of a sample with residual squamous cell carcinoma (SCC) are presented in Figure 3. False color and thresholded cross-polarized terahertz images are shown in Figures 3 a and b, respectively. In Figure 3 b, terahertz image is presented as black and white binary map, where black color corresponds to the areas classified as tumor with normalized terahertz reflectivity values lower than 0.17. Optical cross-polarized and PLI images, as well as corresponding digitized H&E histopathology are presented in Figures 3 c, d, and e, respectively. The cancer is outlined with a dashed line in Figure 3 e. Tumor areas can be clearly identified in terahertz (Figs. 3 a and b) and optical images (Figs. 3 c and d). However, even though the contrast of the terahertz image in Figure 3 a is higher, as compared to optical images, the actual shape of the tumor is better visualized in the optical images (Figures 3 c and d), which correlates well with histology. It can also be appreciated that several black areas, which indicate tumor in the thresholded terahertz image, along the rim of the specimen (Figure 3 b) are not confirmed by the gold standard of histopathology (Figure 3 e). This indicates that some terahertz pixels were misclassified.

To enable higher resolution inspection of these pixels, we examined corresponding areas in optical images outlined with squares in Figure 3 and compared them with respective areas in histopathology. The results are presented in Figures 4 and 5. The false positive area at the bottom of the image in Figure 3 b is outlined with solid line and magnified in Figure 4. Detailed examination of optical superficial PLI (Figure 4 a) and cross-polarized images (Figure 4 b) reveals normal skin structures, such as epidermis (dotted

arrow), dermis (solid arrow), hair follicles (dash and dotted arrows) and fat (dashed arrow). Both optical images correlate closely with respective histopathology shown in Figure 4 c.

The area to the left of the cancer nodule that shows up as false positive, is outlined with a dashed square in Figure 3, and is magnified in Figure 5. Similarly, it does not present the structural loss that indicates tumor in the optical images. It reveals normal epidermis (dotted arrow), dermis (solid arrow), fat (dashed arrow) and a lot of hair follicles (dash and dotted arrow).

Low terahertz reflectivity at the epidermal borders of the specimen (Figure 3 a and b) can be explained by uneven thickness of the excisions, which are usually thinner towards epidermal edge. The gaps between tissue and cover slip are filled with saline solution, which increases absorption of terahertz radiation.

In contrast with terahertz images, optical images exhibit sufficient resolution, similar to that of histopathology. They enable identification of false positive and false negative spots. In this manner, all false negative and false positive pixels in the terahertz image were reclassified by a qualitative comparison with the overlaid PLI and cross-polarized optical images. If a region demarcated by terahertz imaging as tumor was also identified by the optical images as an area which exhibited a loss of normal morphological structure, it was classified as tumor. If, on the other hand, the region did not show a loss of structure and exhibited features of normal skin components, such as epidermis, dermis, subcutaneous fat, hair follicles, etc., it was reclassified as normal. Thus using the optical imaging we were able to convert false positive pixels to true negatives and false negatives to true positives based on qualitative high resolution morphological information.

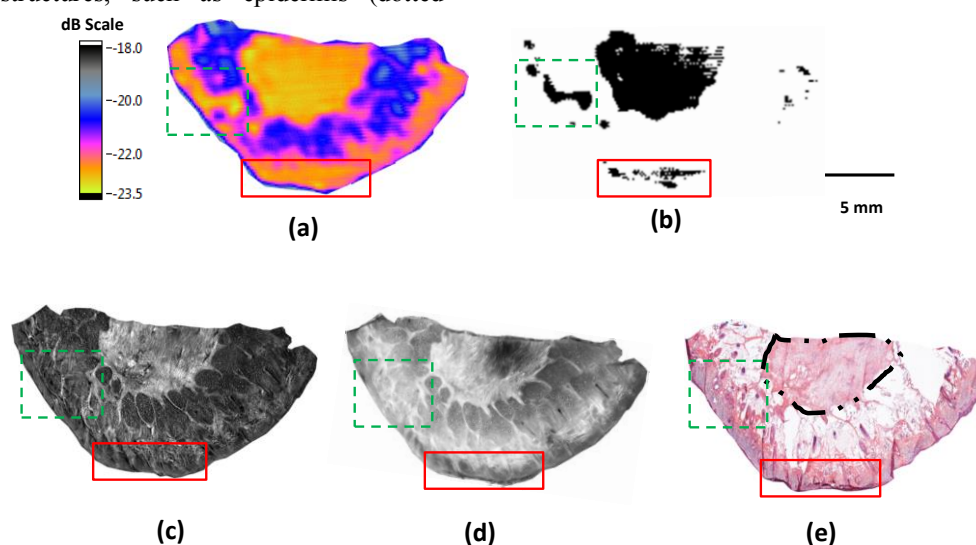


Fig. 3 Images of a skin sample with squamous cell carcinoma. The areas within solid and dashed squares are magnified in figures 4 and 5, respectively. (a) Terahertz image. (b) Thresholded terahertz image. (c) Superficial polarized light optical image (PLI). (d) Cross-polarized optical image. (e) Histopathology with tumor, outlined by dash-and-dot line.

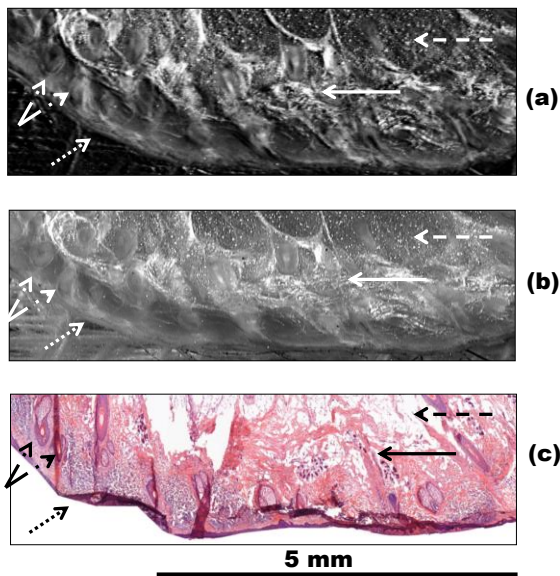


Fig. 4 Images of a skin specimen with SCC. Solid arrow points to collagen, dashed arrow to fat, dotted arrow to epidermis, dash and dotted arrows to hair follicles. (a) Superficial polarized light optical image (PLI). (b) Cross-polarized optical image. (c) Histopathology.

Our results, shown in Figures 3-5, demonstrate that terahertz and optical images offer complementary information that can potentially be used for the accurate assessment of skin cancer margins. In particular, terahertz imaging provides intrinsic contrast, which helps to locate areas suspicious for cancer without extrinsic contrast agents. Our proposed method of quantitative analysis of the terahertz images relies on thresholding of normalized cross-polarized remittance from the sample to highlight areas suspicious for tumor. Thus, areas of low cross-polarized reflectivity may indicate cancerous areas, while higher reflectivity values indicate normal tissue. However, as can be seen

in Figure 3, simply applying a threshold to the image is insufficient for margin delineation, false positive (dark areas near sample edges) and false negatives (white areas within tumor boundary) also occur and the resolution of the terahertz image is insufficient to make out any morphological information that could resolve this. Optical imaging has an advantage of higher resolution that helps with accurate assessment of tissue morphology in the areas highlighted by terahertz imaging. As can be appreciated in Figures 4 and 5, combining information from the optical and terahertz images allows for reduction and in some cases, complete elimination of false positives and false negatives by re-examining areas characterized by low reflectivity in terahertz image. Thus the terahertz image serves as a beacon to localize potential tumor sites, while optical imaging maps tissue morphology and is used for accurate and precise margin delineation, as well as for eliminating artifacts and false positives. A characteristic feature of the proposed multimodal technique, potentially important for future real-time implementation, is a considerable economy of time and computational resources achieved through selective optical analysis of only cancer-suspicious terahertz pixels.

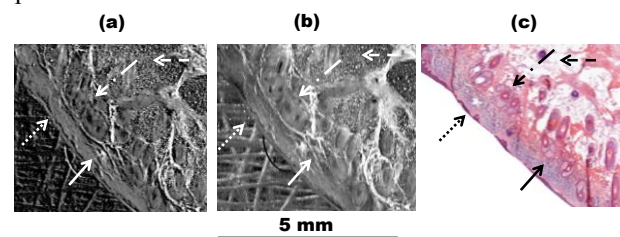


Fig. 5 Images of a skin specimen with SCC. Solid arrow points to collagen, dashed arrow to fat, dotted arrow to epidermis, dash and dotted arrow to hair follicles. (a) Superficial polarized light optical image (PLI). (b) Cross-polarized optical image. (c) Histopathology.

Table 2 Sensitivity and Specificity of Terahertz and Terahertz + Optical Methods.

Specimen #	# of Statistical Data Points		Terahertz Imaging		Terahertz + Optical Imaging	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
1	150	474	90.67	97.47	100	99.16
2	184	440	77.17	94.09	98.91	100
3	132	444	100	97.75	100	99.1
4	8	136	100	98.53	100	100
5	142	298	61.97	97.99	94.37	100
6	78	468	66.67	92.74	87.18	96.15
7	104	536	92.31	90.3	94.23	99.25
8	68	252	97.06	96.03	97.06	99.21
9	218	258	56.88	90.7	97.25	97.67
10	138	318	78.26	88.05	91.3	94.97
Overall			82.1	94.4	96.03	98.55

For statistical evaluation, we have determined the sensitivity and specificity of terahertz imaging alone. Then the areas positive for cancer in terahertz images were re-evaluated based on the correlation between optical images and histopathology. The results of statistical analysis for the sample presented in Figures 3, 4 and 5, show that the sensitivity and specificity of terahertz imaging alone is 82% and 94%, respectively. Combined optical and terahertz image analysis improved the sensitivity and specificity to 96% and 99%, respectively. McNemar's test confirmed that the two methods, i.e. terahertz and combined optical terahertz imaging, are significantly different with a $p < 0.0001$. In total we have imaged and analyzed 10 samples. The results of statistical analysis performed for each sample are presented in table 2. In all the cases investigated, the combination of optical and terahertz imaging yielded highly significant improvement on the results of terahertz imaging alone. These results are comparable to those yielded by contrast enhanced polarization optical imaging. [6-8, 17]. However, the technique presented in this paper does not require the use of exogenous contrast agents, which could potentially cause adverse phenomena such as allergic reactions.

The analysis of the results of the entire set of images collected in the study using a generalized linear mixture model, which took into consideration the inherent correlation within each sample and the pairing of the observations, demonstrated that the difference between terahertz and combined optical / terahertz imaging was statistically significant with $p\text{-value} < 0.0001$. It has also confirmed that the combined optical and terahertz imaging yielded more predictive results, i.e., provided better correlation with the gold standard of histopathology.

It should be noted that in its current implementation the terahertz imaging system cannot be deployed in clinic. However, we plan to develop a combined terahertz and optical system for imaging surgical bed during skin cancer surgeries that would offer effective tumor delineation without the need for exogenous contrast agents.

4 Summary

In this paper we presented experimental evaluation and statistical comparison of two methods, i.e., cross-polarized continuous-wave terahertz imaging and multimodal polarization-sensitive optical and terahertz approach, which can be termed on-demand optical-over-terahertz imaging, for the intraoperative *ex vivo* delineation of NMSC.

We have tested our approach to combining diagnostic information yielded by the terahertz and optical imaging, where terahertz image is used to reveal potential tumor sites, while morphological analysis of the optical images is confined to the areas highlighted by the terahertz approach. This allows for effective elimination of the false positives and leads to an optimized cancer delineation procedure achieved through selective optical analysis of only cancer-suspicious terahertz pixels.

We determined the sensitivity and specificity of the terahertz only and multimodal methods using generalized linear mixture model. To the best of our knowledge, this is the first report of the sensitivity and specificity of the terahertz and multimodal terahertz-PLI imaging for skin cancer demarcation. Statistical analysis revealed that combined optical PLI and terahertz imaging is significantly different and yields significant improvement of sensitivity and specificity, as compared to the terahertz imaging alone.

Our results indicate that combined on-demand optical-over-terahertz imaging of skin cancer has the potential to provide accurate and rapid intraoperative margin delineation during skin cancer removal, potentially eliminating the need for histology processing at every stage of the surgical procedure, thereby reducing time, labor and cost of treatment. Further *in vivo* studies would be the next step towards realizing this goal.

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Laser photochemistry of oxygen. Application to studies of the absorption spectra of dissolved oxygen molecules

Alexander Krasnovsky^{1,2*}, and Anton Kozlov¹

¹ Federal Centre for Biotechnology, A.N. Bach Institute of Biochemistry, Russian Academy of Science, 33 Leninskii pr., Moscow 119071, Russian Federation

² M.V. Lomonosov Moscow State University, Department of Biology, Vorobyevy gori, Moscow 119899, Russian Federation

* e-mail: phoal@mail.ru

Abstract. This paper summarizes the data of our lab on the rates of photooxygenation of singlet oxygen traps upon direct laser excitation of oxygen in air-saturated organic solvents and water. Methods of application of these data to calculation of absorbance (A) and molar absorption coefficients (ϵ) in the maxima of the main oxygen absorption bands (1273, 765 and 1070 nm) are discussed. The most accurate results were obtained from comparing the photooxygenation rates upon porphyrin-photosensitized and direct excitation of oxygen molecules. It is shown that ϵ_{1273} is not sensitive to the presence of heavy atom (bromine) in solvent molecules and markedly decreases on going from non-polar solvents to water being proportional to the radiative rate constants obtained from the quantum yields of singlet oxygen phosphorescence at 1274 nm. The coefficient ϵ_{765} markedly increases in the presence of bromine. In solvents lacking heavy atoms the 1.5-2-fold increase of ϵ_{765} was observed on going from non-polar solvents to water. Simultaneously, the ratios $\epsilon_{1273}/\epsilon_{765}$ are changed from (7-10)/1 in non-polar solvents to 1.5/1 in water. The value of ϵ_{1070} obtained in carbon tetrachloride is shown to be about two orders smaller than ϵ_{1273} in the same solvent. The results are important for both analyses of oxygen photonics and dosimetry of laser radiation in biomedical experiments. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Molecular oxygen; singlet oxygen; photosensitizers; direct oxygen excitation.

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1 Introduction

It has been known since the seminal papers of R. Mulliken that oxygen molecules have triplet ground state and two relatively low-lying singlet levels [1,2]. Fig. 1 shows energy diagram of the major electronic transitions in monomeric oxygen molecules in rarified gas as follows from measurements of the absorption spectra in Earth atmosphere and in the gas phase at atmospheric pressure [3-6]. These transitions are highly forbidden therefore the absorption bands of monomeric oxygen molecules are so weak that they normally cannot be measured using conventional spectrophotometers under ambient conditions.

Reliable absorption spectra were obtained in the gas phase and solutions under high oxygen pressure of 50-140 atm and in liquid oxygen. However, under these conditions dimerization of oxygen molecule occurs, therefore the absorption spectra correspond mainly to dimols (O_2)₂. In the dimol spectra, the IR maxima analogous to those of monomols are shifted toward shorter wavelengths, and a series of additional maxima appear in visible region. Relative intensities of the spectral peaks of dimols are strongly different from those in the gas phase at atmospheric pressure [6-11] (Fig. 2).

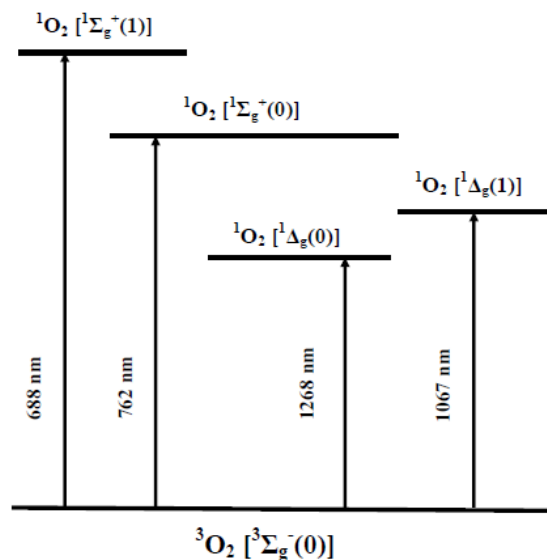


Fig. 1 The main electronic transitions in monomeric oxygen molecules in the rarefied gas as reported in refs [3-6]. The vibrational sublevels are shown in brackets.

It was reported in the classic papers by Evans [7] and Matheson's group [12,13] that excitation of oxygen dimols dissolved in organic solvents at oxygen pressure 130-140 atm. leads to formation of the $^1\Delta_g$ singlet oxygen, which causes oxygenation of added organic

singlet oxygen traps, aminoacids, proteins and other molecules.

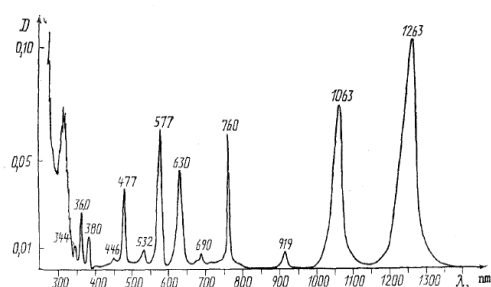


Fig. 2 Absorption spectrum of oxygen dimols in compressed oxygen at 150 atm. in the 5 cm optical path [10,11].

Based on these experiments, Ambartzumian and his collaborators advanced an idea that direct laser excitation of intrinsic dimol and monomol oxygen molecules in living cells might be a reason for biological action of laser radiation in visible and infrared [14,15]. It was also suggested that laser excitation of oxygen might be applied to destruction of cancer cells in vitro and in animal and human organisms.

These papers caused heated discussions. Apparently, biological action caused by laser excitation of oxygen molecules should be directly proportional to the rate of $^1\text{O}_2$ production by laser radiation, which in turn, depends on laser power (I_{las}) and the fraction of laser light absorbed by oxygen molecules ($1 - 10^{-A_{\text{ox}}}$), where A_{ox} is oxygen absorbance ($A_{\text{ox}} \ll 0.01$). Hence:

Biological effect ~

$$\sim I_{\text{las}} (1 - 10^{-A_{\text{ox}}}) = I_{\text{las}} \times 2.3 \times A_{\text{ox}}. \quad (1)$$

Although biological and therapeutic action of red and IR lasers has been reported many times ([15-19] and refs therein) there is no crucial evidence showing that this action under ambient conditions and moderate (therapeutic) laser power is always due to oxygen excitation. Thus, reliable information on absorption coefficients of oxygen molecules under normal conditions is of great importance for both understanding of oxygen photonics in natural environment and mechanistic analyses of laser action and dosimetry of IR laser radiation in biomedical experiments.

Several years ago our group started work on mimicking biological effects of laser radiation in solutions of singlet ($^1\Delta_g$) oxygen traps in organic solvents and water at normal pressure and temperature. It was established that monomeric oxygen molecules dissolved in these solvents work as photosensitizers of oxygenation reactions. Kinetic analyses of the reaction rates allowed us to develop methods for calculation of absorbance (optical density) for oxygen absorption bands under natural conditions. The present paper summarizes major results of our group [20-28].

2 Experimental procedure

For sample irradiation, diode lasers LAMI Gelios ("Surgical innovation technologies", Moscow) were applied with the maxima of the emission bands at 1267-1270, 1064-1070 and 760-762 nm and the half bandwidth 4-12 nm. Laser light was focused into a fiber light guide with of 1 mm diameter. The eliciting light power, was controlled by the power meter Ophir ORION-TH with the sensor head 20C-SH (Israel). Solutions were irradiated in the 10 mm rectangular quartz cell under normal pressure and room temperature. Volume of the solutions was 1.5 ml. Diameter of the irradiated spot was 5-8 mm.

Tetracene, (Aldrich Chemistry, >98%), 1,3-diphenylisobenzofuran (DPIBF) and uric acid (Acros Organics) were used as singlet oxygen traps. Tetraphenylporphyrin (TPP) (Aldrich, Chemistry) was employed in certain experiments as photosensitizers of singlet oxygen production. Absorption spectra were measured using a Hitachi U-3400 (Japan) and SF-56 (LOMO Spektr, St. Petersburg) spectrophotometers. The rate of $^1\text{O}_2$ formation was calculated from the rate of photobleaching of the main absorption maxima of the traps (474 nm for tetracene, 410-414 nm for DPIBF, 530 nm for rubrene and 290 nm for uric acid) under irradiation by IR lasers.

It was shown that irradiation of air-saturated solutions of the traps in organic solvents and water by IR lasers of moderate power (0.03-2 W) with the emission maxima at 1270, 1070 and 765 nm causes oxygenation of the traps, which is accompanied by bleaching of their main absorption bands. The oxygenation rates were measured as follows. At first, the absorbance (optical density) (A_0) in the main absorption maximum of a trap was recorded. In most experiments, solutions with $A_0 = 0.8-1.5$ in the 1 cm cell quartz were employed. Then, the solutions were irradiated during 1-30 min, shaken, and the absorbance (A_f) was measured again. The photoreaction rates (V_{lasr} , $\Delta A/\text{min}$) were calculated from measuring the illumination time (t) needed to decrease the absorbance so that $\Delta A = A_0 - A_f = 0.05 - 0.1$. As a result, not more than 15% overall trap content was oxidized during irradiation. The oxygenation rates were then normalized to power of laser radiation in einstein/s (see details in refs [20-28]).

2.1 Mechanistic analyses

The photooxygenation action spectra were measured using tunable lasers. These spectra indicate that oxygenation of the traps occurred due to direct laser excitation of oxygen molecules. In CCl_4 , C_6F_6 and ethanol the main IR absorption maximum was found at 1273 nm. The width (FWHM) of this band was 17-18 nm (106 cm^{-1}) [20,21,23] (Fig. 3). This maximum is shifted by 5 nm to longer wavelengths compared to the absorption maxima of monomeric oxygen in rarified gas (Fig. 1). More recently these results were confirmed by

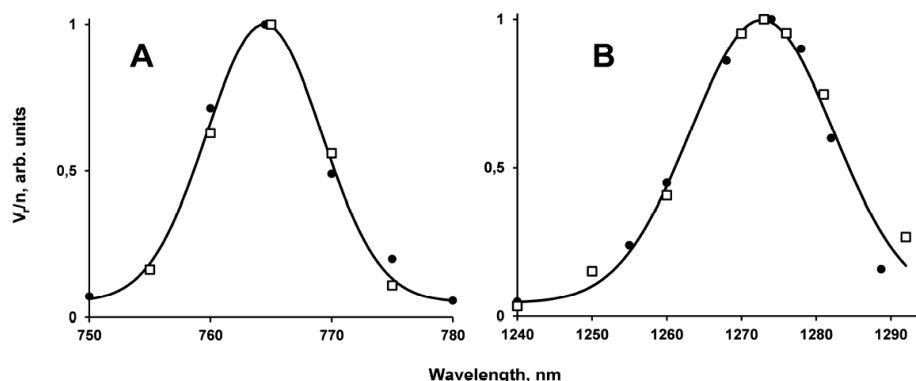
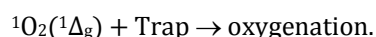
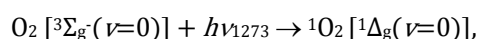
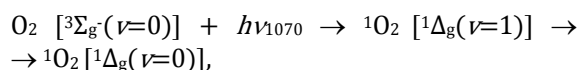
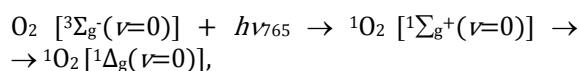


Fig. 3 Action spectra of oxygenation of the singlet oxygen traps under direct excitation of oxygen molecules by IR laser radiation. A: spectra obtained using tunable titan-sapphire laser in solutions of tetracene (•) [24] and 1,3-diphenylisobenzofuran (□) in CCl_4 [26]. B: spectra obtained using tunable forsterite laser in solutions of tetracene in CCl_4 (•) [21] and 1,3-diphenylisobenzofuran in ethanol (□) [23]. V_r is the rate of trap photooxygenation, n is irradiation intensity in number of photons per second.

Courtade's group in France who observed the oxygen excitation band at 1273 nm in CCl_4 , acetone, ethanol and D_2O with FWHM ~ 15 nm [29,30].

The action spectra in the dark red region were reported for the first time in papers of our group [24,26] (Fig. 3). In CCl_4 , C_6F_6 and ethanol the maximum was found at 765 nm, at slightly longer wavelengths than in the rarefied gas (Fig. 1). The band width was estimated to be 8-9 nm (~ 130 cm^{-1}). Recently Ogilby's group in Denmark confirmed main results of our studies using luminescence technique and extended measurements to several additional solvents. In the luminescence experiments of this group the excitation maxima varied within 763-772 nm and FWHM -within 6-17 nm. In agreement with our data in CCl_4 they observed the excitation maximum at 765 nm with the bandwidth of 7 nm (120 cm^{-1}) [31]. Apparently, parameters of the action spectra characterize the major absorption bands of dissolved oxygen under ambient conditions.

In organic solvents the oxygenation of the traps was inhibited by singlet oxygen quencher – β -carotene, in water and alcohols – by sodium azide. The photoreaction was not observed after purging with argon or nitrogen. After saturation with pure oxygen the photoreaction rates increased 5-fold compared to air-saturated solutions [20-24]. Thus, evidence was obtained that under ambient conditions, monomeric oxygen molecules work as photosensitizers of oxygenation reactions similarly to oxygen dimers appearing at high oxygen pressure. The mechanisms of the photoreactions correspond to the following scheme:



According to this scheme, photoexcitation of oxygen at 1273 nm causes direct population of the [$^1\Delta_g(v=0)$] (v is a vibrational sublevel) state. Photoexcitation at 1070 nm causes direct population of the [$^1\Delta_g(v=1)$] state and photoexcitation at 765 nm causes population of the singlet level [$^1\Sigma_g^+(v=0)$]. It is known that in the solution-phase [$^1\Delta_g(v=1)$] and [$^1\Sigma_g^+(v=0)$] rapidly (for nano- or picoseconds) lose extra energy and populate the [$^1\Delta_g(v=0)$] state due to collisions of oxygen and solvent molecules ([19-21]). Thus, no matter what level of oxygen is initially populated, chemical activity is determined by the [$^1\Delta_g(v=0)$] state of O_2 .

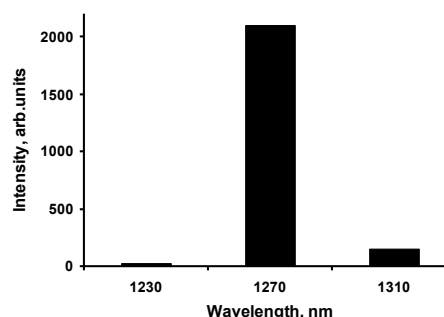


Fig. 4 Spectrum of $^1\text{O}_2$ phosphorescence in air-saturated pigment-free C_6F_6 upon excitation of dissolved oxygen by monochromatic light of cw diode laser at 766 nm. The spectrum was measured using three changeable interference filters transmitting light at 1230, 1270 and 1310 nm respectively. The luminescence data were obtained in collaboration with A.S. Benditkis.

Quite recently we assembled a very sensitive spectrometer, in which phosphorescence of $^1\text{O}_2$ at 1270 nm is detected using cooled S-1 PMT (FEU-112) through three changeable interference filters with the transmission maxima at 1230, 1270 and 1310 nm. In

agreement with the above scheme and the data of Ogilby's group [31] we observed singlet oxygen phosphorescence at 1270 nm in air-saturated CCl_4 and C_6F_6 under excitation by cw laser at 765 nm (900 mW) without pigments-photosensitizers. First results are shown in Fig. 4. No light emission was observed upon excitation at 740 nm. Addition of acetone (50%) caused the decrease of luminescence intensity by more than two orders. Under oxygen purging the luminescence intensity increased (more detailed paper is in preparation).

2.2 Principles of data analysis

Based on this mechanistic scheme, the following equation was proposed for the rate (V_{lasr} , M/s) of the trap photooxygenation under laser excitation of oxygen [27-28]:

$$V_{\text{lasr}} = 2.3 A_{\text{max}} I_{\text{las}} \alpha k_{\text{ox}} [\text{Trap}] / (k_{\Delta} + k_{\text{ox}} [\text{Trap}]), \quad (2)$$

where A_{max} is absorbance (optical density) in the maxima of the oxygen absorption spectrum, I_{las} is the incident photon flux of IR laser radiation in $\text{einstein} \times \text{L}^{-1} \text{s}^{-1}$, k_{ox} is the rate constant of oxygenation of the traps by singlet oxygen; $[\text{Trap}]$ is the molar concentration of the trap; k_{Δ} is the rate constant of the $^1\text{O}_2$ deactivation in the trap-free solutions, i.e. $k_{\Delta} = 1/\tau_{\Delta}$ where τ_{Δ} is the lifetime of singlet oxygen in solvents lacking the singlet oxygen traps.

The coefficient α corresponds to degree of overlapping of the action spectrum with the spectrum of laser radiation. It was estimated from the photooxygenation action spectra (Fig. 3) and laser emission spectra using the "Origin" program:

$$\alpha = \int A(\nu) I(\nu) d\nu / \int I(\nu) d\nu, \quad (3)$$

where $A(\nu)$ is a function characterizing normalized excitation spectrum of oxygen (Fig. 2) in energy units, in which A varies from 0 to 1; and $I(\nu)$ is a function characterizing the normalized spectrum of laser radiation, where I varies from 0 to 1. Apparently, if the wavelength of laser light exactly corresponds to the maximum of the oxygen absorption band, α is equal to 1. Usually, the emission maxima of diode lasers, were shifted to shorter wavelengths compared to the oxygen absorption maxima, therefore α varied from 0.2 to 0.9 (see [27,28] for details).

Eq. 2 allows for accurate determination of the oxygen absorption coefficients corresponding to the maxima of oxygen absorption bands using experimental values for the oxygenation rates under laser irradiation:

$$A_{\text{max}} = V_{\text{lasr}} (k_{\Delta} + k_{\text{ox}} [\text{Trap}]) / 2.3 \alpha I_{\text{las}} k_{\text{ox}} [\text{Trap}]. \quad (4)$$

Knowing A_{max} , one can calculate molar absorption coefficients of oxygen $\epsilon_{\text{max}} = A_{\text{max}} / [O_2]$, where $[O_2]$ is the molar concentration of oxygen in solutions. The

absorption cross section $\sigma_{\text{max}} = 2300 \epsilon_{\text{max}} / N_{\text{av}}$ where N_{av} is Avogadro number.

2.3 Absorbance at 1273 nm

The first paper, in which A_{1273} was estimated using Eq. 4 and air-saturated carbon tetrachloride as a solvent, was published in 2004 [25]. The data were obtained from measurement of the oxygenation rates of tetracene upon excitation of dissolved oxygen at 1273 nm by tunable forsterite laser. The authors arrived to the values: $A_{1273} \approx 7.2 \times 10^{-6}$, $\epsilon_{1273} \approx 0.003 \text{ M}^{-1} \text{cm}^{-1}$, $\sigma_{1273} \approx 10^{-23} \text{ cm}^2$, which served us as a reference during several years. Because of technical limitations higher precision could not be reached at that time. Then, accurate measurements of the relative rates of DPIBF oxygenation in different solvents were carried out. The results allowed analysis of the solvent dependence of the oxygen absorption coefficients. The first paper on this subject was published in 2005 [22]. Later, more detailed papers followed [23,25]. In particular, it was established that in accord with the Einstein law, the molar absorption coefficients corresponding to the oxygen absorption band at 1273 nm in series of organic solvents were proportional to the radiative rate constants (k_r) for this electronic transition obtained from measurement of the quantum yield of singlet oxygen phosphorescence (n is the refractive index) [22-25]:

$$\epsilon_{1273} \sim k_r / n^2. \quad (5)$$

However, in the papers mentioned above the proportionality was not revealed in water and alcohols. We proposed that this effect might be due to problems in kinetic analyses of the data [26,27]. Indeed, in the first papers we assumed that the rate constant (k_{ox}) for the reaction of the trap with $^1\text{O}_2$ and molar absorption coefficients of traps (ϵ_{trap}), which were used for determination of the trap concentrations, did not depend on solvents. In order to solve this problem an experimental procedure was developed, which excluded ϵ_{trap} and k_{ox} from calculations. To reach this goal, the rates of oxygenation of the traps were compared upon direct and photosensitized oxygen excitation at equal trap concentrations. In this case, the following equation was obtained [26-28]:

$$A_{\text{max}} = (V_{\text{lasr}} / V_{\text{psr}}) (I_{\text{psex}} / \alpha I_{\text{las}}) \Phi_{\Delta} (1 - 10^{-A_{\text{ps}}}) / 2.3, \quad (6)$$

where V_{psr} is the rate of photosensitized oxygenation of the traps; I_{las} and I_{psex} are the incident photon flux in $\text{einstein L}^{-1} \text{s}^{-1}$ for light applied to excitation of oxygen and photosensitizer respectively; Φ_{Δ} is the yield of singlet oxygen generation by pigments photosensitizers (TPP and TPPS were employed in our experiments). For calculations with Eq. 6, the photooxygenation rates were measured in the $\Delta A/t$ units, where ΔA is a change of absorbance in the maxima of the absorption spectra of the traps and t is time (minutes) of laser irradiation. Instead, one has to know accurate values of Φ_{Δ} for

Table 1 Values of the oxygen absorption coefficients in several solvents calculated according to Eqs. 4 and 6, using the rates of oxygenation of the singlet oxygen traps under ambient conditions.

	$A_{1273} \times 10^5$ (1 cm)		$A_{765} \times 10^5$ (1 cm)	[O ₂], mM [32]	$\varepsilon_{1273} \times 10^3$, M ⁻¹ cm ⁻¹	$\varepsilon_{765} \times 10^3$, M ⁻¹ cm ⁻¹	$\varepsilon_{765}/$ ε_{1273}	Permit- tivity
	Eq. 4*	Eq. 6**	Eq. 6**					
CCl ₄	-	1.33	0.19	2.6	5.1	0.7	0.14	2.24
C ₆ F ₆	2.25	2.25	0.34	4.4	5.1	0.77	0.15	2.05
C ₆ H ₆	1.2	1.20	0.13	1.9	6.3	0.68	0.11	2.27
Toluene	1.2	1.18	0.12	1.8	6.5	0.67	0.10	2.38
n-heptane	-	0.92	0.15	2.8	3.3	0.53	0.16	1.94
1-Bromo- hexane	-	0.96	0.36	3.0	3.2	1.2	0.38	5.4
Acetone	0.70	0.66	0.20	2.4	2.8	0.83	0.30	20.7
Ethanol	-	0.48	0.14	1.65	2.9	0.85	0.29	24.5
H ₂ O	-	0.043	0.032	0.29	1.5	1.1	0.75	80.1

*The data of this column were obtained using Eq. 4 from comparison of the rates of DPBIF oxygenation in organic solvents and carbon tetrachloride. The absorption coefficients in CCl₄ were measured using Eq. 6 (see [28] and refs therein). **The numbers in these columns (new data of our experiments) were obtained using Eq. 6 from comparison of the oxygenation rates upon direct and photosensitized oxygen excitation in each solvent. Maximum deviation from the average values indicated in this table did not exceed 10%.

photosensitizers. Our experiments show that for monomeric TPP and TPPS in most solvents used in our work, $\Phi_A = 0.73 \pm 0.05$ ([26-28] and refs therein), only in 1-bromohexane we obtained $\Phi_A = 0.92 \pm 0.02$ (unpublished).

This method was applied for the first time in 2012 [26], in which we averaged current and previous results of our group for four solvents: CCl₄, acetone, ethanol and water. The ways to estimate coefficients α were not found at that time therefore it was suggested that $\alpha \approx 1$ in all experiments. It was obtained using this assumption, DPIBF and Eq. 6 that absorbance of oxygen at 1270 nm in CCl₄ is 1.5 times higher than we reported previously [21]. Besides, the relative values of the molar absorption coefficients for this band were proved to be proportional to the radiative rate constants (k_r) of singlet oxygen also in water and ethanol. Deviation from the proportionality in water and alcohol reported in the preceding papers [22,23,25] was shown to be due to the strong increase of k_{ox} for DPIBF in these media compared to organic solvents having no OH-groups (see [26,28] for details).

Papers of 2003-2012 solved principal experimental problems arising upon measurement of the absorption coefficients for dissolved oxygen. As a result, estimation of the numerical values for these coefficients in different environment was carried out [24-26]. As knowledge of the oxygen absorption coefficients is of fundamental importance for oxygen photonics, subsequent experiments were aimed at increasing the accuracy of our measurements.

For this goal, diode lasers having relatively narrow spectral band (4-6 nm) were employed. Coefficients α were calculated from the action spectra of trap oxygenation (Fig. 2) and spectra of laser radiation (in energy units) using Eq. 2 (see [27,28] for details). Two methods of analysis were applied. According to one of

them, A_{1273} was carefully measured in CCl₄ using Eq. 6 and relative rates of oxygenation of three traps – DPIBF, tetracene and rubrene upon photosensitized and direct oxygen excitation [28]. It was established that for all traps the relative oxygenation rates upon photosensitized and direct oxygen excitation normalized to power of exciting radiation and the fraction of light absorbed by porphyrins were $\sim 10^4$:

$$(V_{psr} / V_{lasr}) / (I_{psex} / \alpha I_{las}) \approx 10^4. \quad (7)$$

Hence, if 100% exciting light is absorbed by porphyrins, the efficiency of photosensitized oxygen excitation is 10^4 times higher than the efficiency of direct oxygen excitation. In real experiments porphyrin absorbed not more than 10% exciting light, therefore relative efficiency of photosensitized oxygen excitation was by one order smaller, therefore the ratio corresponding to Eq. 7 is about 10^3 [28].

In addition, it was found that A_{1273} in CCl₄ is almost two times greater than the value reported in ref. [21] (Table 1). The difference was shown to be mainly due to the fact that the real value of the molar absorption coefficient for tetracene was almost two times smaller than the literature value applied in the previous work [21] (see [28] for details). The obtained A_{1273} in CCl₄ was then used for correction of numerous results of our previous measurements of A_{1273} in other organic solvents, which were estimated using Eq. 4 and CCl₄ as the reference solvent [28] (Table 1). As mentioned above, these results were obtained upon direct laser excitation of oxygen from relative rates of DPIBF oxygenation in CCl₄ and other organic solvents having no OH groups in their molecules.

According to the second method, the rates of DPIBF oxygenation were compared upon direct and photosensitized oxygen excitation in each solvent. The

Table 2 Correlation between obtained ε_{1273} and the radiative rate constants (k_r , s^{-1}) in several solvents.

Parameters	CCl ₄	C ₆ F ₆	n-heptane	Benzene	Toluene	Acetone	Ethanol	H ₂ O
$\varepsilon_{1273} \times 10^3$, $M^{-1}cm^{-1}$	5.1	5.1	3.3	6.2	6.7	2.8	2.9	1.5
ε_{1273} , rel. units	1	1.0	0.65	1.2	1.3	0.55	0.57	0.29
k_r/n^2 , rel. units [23,33]	1	1.0	0.62	1.2	1.2	0.53	0.54	0.25

Table 3 The radiative rate constants (k_r) and the phosphorescence quantum yields (Φ_r) for triplet-singlet transitions in oxygen molecules dissolved in carbon tetrachloride estimated from the experimentally obtained ε_{1273} and ε_{765} (Table 1) using Eq. 8.

Transitions	ν , cm^{-1}	$\Delta\nu$, cm^{-1}	$\varepsilon_{max} \times 10^3$ $M^{-1}cm^{-1}$	k_r , s^{-1}	k_r , $s^{-1}{}^a$	Φ_r
${}^1\Delta_g(0) \leftarrow {}^3\Sigma_g^-(0)$	7855	~ 103	5.1	0.60	1.17; 0.25	0.018
${}^1\Sigma_g^+(0) \leftarrow {}^3\Sigma_g^-(0)$	13072	~ 130	0.71	0.22	0.40; 1.55	2.9×10^{-7}

^aThis column shows the experimentally measured k_r , reported in papers cited in Ref. [26];

^b $\Phi_r = k_r \tau$ where τ is the lifetime of singlet oxygen equal to ~ 30 ms for the ${}^1\Delta_g$ state [37] and 130 ns for the ${}^1\Sigma_g^+$ state in the reagent grade CCl₄ ([38,39] and refs therein), n is 1.46 in CCl₄.

most recent results of our studies are shown in Table 1. As seen from the Table, both methods yield similar values of the absorption coefficients. Correctness of the coefficients ε_{1273} presented in Table 1 is supported by apparent correlation of their relative values with the relative values of k_r/n^2 for the ${}^1\Delta_g$ -state of singlet oxygen in different solvents (Table 2).

Thus, the data of Table 2 indicate that one and the same mechanism is responsible for the solvent dependence of both k_r , calculated from the quantum yield of singlet oxygen phosphorescence at 1270 nm and ε_{1273} obtained by methods of laser photochemistry. Both coefficients are about 1000 times greater than those in rarefied gas (see [21] for refs). It follows from Minaev's theoretical considerations that due to spin-orbit coupling in O₂ molecules and perturbations caused by their collisions with solvents, the $a^1\Delta_g \leftarrow X^3\Sigma_g^-$ transition which is purely magnetic in the rarefied gas becomes allowed as electric dipole in solutions [34,35].

Hence, one can estimate the absolute value of k_r from the obtained ε_{1273} using the equation for the electric dipole transitions (so called, Kravetz equation). The simplest form of this equation was provided by Strickler and Berg [36]:

$$k_r = n^2 2.88 \times 10^{-9} \nu^2 \Delta\nu \varepsilon_{ox} g_1 / g_2, \quad (8)$$

where n is the solvent refractive index, ν is the transition frequency in cm^{-1} , $\Delta\nu$ is the half-width of the oxygen absorption band (FWHM), ε_{ox} is the molar absorption coefficient of oxygen in $M^{-1}cm^{-1}$ and g_1 and g_2 are multiplicity of the initial and final states, $g_1/g_2 = 3/1$ (see also [26] and refs therein). Table 3 shows the results of application of Eq. 8 to the parameters of oxygen obtained in CCl₄ (Table 1) and compares these results with the experimentally measured values obtained by different groups. Taking into account an

approximate character of Eq. 8 and scattering of the experimental numbers, correlation between the calculated and measured k_r is satisfactory.

2.4 Absorbance at 765 nm

The first paper, in which A_{765} was estimated in air-saturated carbon tetrachloride was published in 2007 [24]. Tetracene was used as the singlet oxygen trap and the tunable titan-sapphire laser was applied for oxygen excitation. The maximum of the photooxygenation action spectrum was observed at 765 nm (Fig. 3); A_{765} was roughly estimated to be 3.5 times smaller than A_{1273} and $\varepsilon_{765} \approx 10^{-3} M^{-1}cm^{-1}$ was obtained [24]. Later, similar measurements were performed using DPIBF as a singlet oxygen trap. Values of A_{765} were estimated in carbon tetrachloride, acetone, ethanol and water. It was established that $\varepsilon_{765} \approx 10^{-3} M^{-1}cm^{-1}$ in these solvents [26]. After further improvement of the measurement procedure and data analyses with accounting for coefficients α (see previous section) it was found that ε_{1273} is almost two times greater than that obtained previously and the ratio ($\varepsilon_{1273}/\varepsilon_{765}$) was equal to ~ 7 in CCl₄. A slight increase of ε_{765} with the increase of solvent polarity was also reported [27,28].

The most recent studies were performed based on Eq. 6 and comparison of the rates of DPIBF oxygenation upon direct and photosensitized oxygen excitation. In these experiments we used a new diode laser with the emission maximum at exactly 765 nm and 2.5 nm bandwidth. Several additional solvents were studied. Some results are presented in Table 1 and Fig. 5. It is seen that the ratio ($\varepsilon_{765}/\varepsilon_{1273}$) varies within 0.1-0.15 in non polar solvents, increases with the increase of solvent polarity to about 0.3 in acetone and ethanol and to 0.75 in water. Exclusion is 1-bromohexane, which despite low polarity, shows relatively high ε_{765} . This

effect can be attributed to the presence of heavy atom (bromine), which is known to enhance the $^1\Sigma_g^+(v=0) \rightarrow ^3\Sigma_g^-(v=0)$ transition [9,31]. Based on these values of k_r , the quantum yield of 1O_2 phosphorescence at 765 nm can be estimated. Table 3 indicate that even in CCl_4 the phosphorescence yield of this emission is very low. In other organic solvents and water the lifetime of $^1\Sigma_g^+$ is known to be much smaller. For instance, it was estimated that $\tau_\Sigma \approx 30$ ps in ethanol and 6.5 ps in water [38,39]. Therefore, the quantum yield of phosphorescence in these solvents should be by ~ 4 orders smaller than in CCl_4 , being about 10^{-11} .

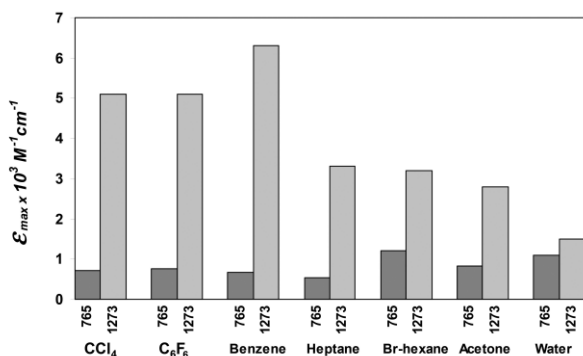


Fig. 5 Molar absorption coefficients corresponding to the main absorption maxima of molecular oxygen in several solvents.

2.5 Absorbance at 1070 nm

The absorption coefficient for the band at 1073 nm corresponding to the transition $^1\Delta_g(v=1) \leftarrow ^3\Sigma_g^-(v=0)$ was reported in Refs. [26,28], in which oxygenation of tetracene and DPIBF solutions were studied under irradiation by the Nd-Yag laser (1064 nm) or by powerful diode lasers (1060 nm). As a result, we arrived to the ratio $A_{1273}/A_{1073} = 60 \pm 15$. Fig. 6 illustrates relative intensities of the major oxygen absorption bands in CCl_4 .

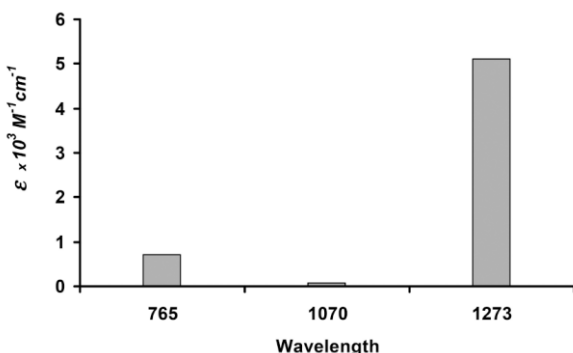


Fig. 6 Relative values of the molar absorption coefficients in the maxima of the absorption spectrum of oxygen dissolved in CCl_4 under normal conditions.

Comparison with Fig. 2 shows that the ratio A_{1073}/A_{1273} obtained in our experiments is 30-40 times smaller than in the absorption spectra of oxygen dimols (O_2)₂ (see

also [9,11,26]). Judging by the ratio A_{1073}/A_{1273} , the concentration of oxygen dimols is very low under normal conditions being less than 3%. However, the ratio A_{1073}/A_{1273} is a mirror image of the intensities of the major bands in the phosphorescence spectrum of monomeric singlet oxygen at 1274 and 1590 nm I_{1590}/I_{1274} (see refs in [26,28]).

3 Conclusions

Thus, application of the methods of laser photochemistry allowed measurement of the absorption coefficients for major absorption bands of monomeric oxygen molecules dissolved in organic solvents and water under ambient conditions. This information has never been reported before our studies. Knowledge of these coefficients is obviously of basic importance for photonics of triplet and singlet oxygen because it provides information on the electronic structure of oxygen molecules and their interaction with environment. Recently, major results of our studies were confirmed by other researchers [29-31]. Although at this state of research numerical values reported by different groups sometimes do not quite coincide (only in acetone identical values were obtained by all authors) the reported values are still of the same order of magnitude. The difference does not exceed the factor of 3. As mentioned in the present paper and previous papers of our group, the values reported by our group also passed through certain evolution due to improvement of measurement techniques and data processing. As mentioned above, to obtain correct numbers one should take into account many parameters, determination of which is not always simple.

Biomedical importance of this research is also apparent. Our experiments demonstrate that direct laser excitation of oxygen molecules is a real natural process, which can be detected using modern laser and spectroscopic techniques. However, the effectiveness of this process is very low, because the absorption coefficients of oxygen have a very small value. The rate of photosensitized singlet oxygen production is 10^3 - 10^4 times greater than the rate of direct oxygen excitation. Therefore, it is difficult to expect that under moderate laser power allowed for photodynamic and laser therapy direct excitation of free oxygen dissolved in cell structures causes appreciable destruction of living tissues. It was shown recently that reasonable rates of cell damage can be observed under laser power of 200 W/cm² that greatly exceeds the excitation power allowed for medical applications (usually < 200 mW/cm²) [40]. However, low-power laser radiation might influence enzyme-bound oxygen molecules whose concentration is much higher. The data are reported that singlet oxygen produces signaling effect, triggers expression of antistress genes and apoptosis and stimulates immune system response [41-44]. In conclusion it is worth noting that according to our results, the lasers with the wavelength 765 nm are probably more appropriate for oxygen excitation in biomedical systems than lasers emitting at 1273 nm.

Indeed, both lasers have rather similar efficiency. However, the dark red light penetrates deeper and causes less heating (see [28] for more details).

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Probing depth in diffuse optical spectroscopy and structured illumination imaging: a Monte Carlo study

Daria A. Loginova^{1,2*}, Ekaterina A. Sergeeva¹, Ilya I. Fiks¹, and Mikhail Yu. Kirillin¹

¹ Institute of Applied Physics RAS, 46 Ulyanov St., Nizhny Novgorod 603950, Russia

² Lobachevsky State University of Nizhny Novgorod, 23 Gagarin ave, Nizhny Novgorod 603950, Russia

* e-mail: vekyf@inbox.ru

Abstract. Diffuse optical spectroscopy (DOS) and its modification employing structured illumination are widely used in monitoring biotissue oxygenation. In such measurements it is important to know the probing volume for definite source-detector configuration; however, it cannot be measured directly. Monte Carlo simulations allow to trace the probing depth of each individual photon contributing to the signal, which provides a numerical solution for this problem. In this study we investigate distributions of photons over maximal depth reached in turbid media (probing depth) with optical parameters typical for cutaneous tissues at the wavelength of 600 nm. Different configurations of probing illumination are considered, such as collimated point source, one-dimension sinusoidal and rectangular patterns. For collimated point source and zero source-detector separation the number of collected photons monotonously decreases with the probing depth while a pronounced maximum in the distribution is manifested with the increase of source-detector separation. The position of this maximum shifts to higher depths with the decrease of μ_a . For one-direction sinusoidal and rectangular illumination patterns it is shown that when the photons are collected near the center of a bright stripe, the peak of the distribution remains close to the surface. When the photons are collected near the center of a dark stripe the peak shifts towards higher depths with the decrease in spatial duty cycle and spatial frequency of the illumination pattern. Employment of rectangular illumination pattern seems more efficient for DOS applications due to wider abilities for controlling probing depth. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Monte Carlo simulations; diffuse optical spectroscopy; structured illumination; spatially modulated light; probing depth.

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1 Introduction

Diffuse optical spectroscopy (DOS) is a modern biomedical diagnostics technique based on registration of scattered spectra for a single or several pairs of source and detectors. Reconstruction of absorption spectra from these measurements allows to evaluate chromophore content within the studied tissue such as oxy- and deoxyhemoglobin, lipid and water as well as to retrieve hemoglobin oxygen saturation index [1,2]. DOS is a perspective tool for a broad range of medical diagnostics tasks including breast cancer diagnostics [4-6], human brain functional diagnostics [7], monitoring of tumor in laboratory animals in experimental oncology [8].

Numerical models of DOS signals are usually based on diffusion approximation of transport theory [9]. It is well known that in reflectance mode typical photon trajectories from source to detector form so-called banana-shaped zones which characterize the most sensitive volume to locate inclusions in the medium ("measurement volume"). Maximum depth of the most probable photon trajectory can be considered as the probing depth of the particular source-detector configuration. It is clear that the probing depth and the measurement volume depend on tissue optical properties and measurement conditions. Although the probing depth and the measurement volume are the characteristics of great importance for clinical applications, they hardly can be determined experimentally. To our knowledge, analytical evaluation of probing depth has been performed only for the simplest source-detector configuration and is unavailable in the case of complex illumination modalities. In this respect Monte Carlo technique is

more appropriate for estimation of the measurement volume and the probing depth. Based on modeling of random photon walk this approach provides information about exact trajectory of an individual photon in the studied medium and allows to analyze the total set of photons trajectories contributing to DOS signal for particular source-detector separation. For instance, Monte Carlo simulations of photon trajectories from source to detector are carried out in studies [10] for semi-infinite medium with a plane boundary and [11] for complex multilayered medium model and demonstrate banana-shaped zone of typical photon trajectories.

The modification of DOS employing structured illumination can be efficiently used for mapping of biotissue absorption coefficient [2,12,13]. Variation of illumination pattern, in particular, changing its spatial frequency, potentially allows to control measurement volume. At the same time, in this modality knowing probing depth is also of high importance.

In this study we report on results of Monte Carlo study of probing depth for DOS and its modality with structured illumination.

2 Materials and methods

2.1 Simulated measurement configurations

Monte Carlo modeling was carried out for a uniform slab sized 10x10 mm in transversal directions and 5 mm in-depth. DOS simulation was performed for two arrangements of the medium illumination: illumination by a collimated point source and illumination by a planar source with one-dimension striped pattern. In the

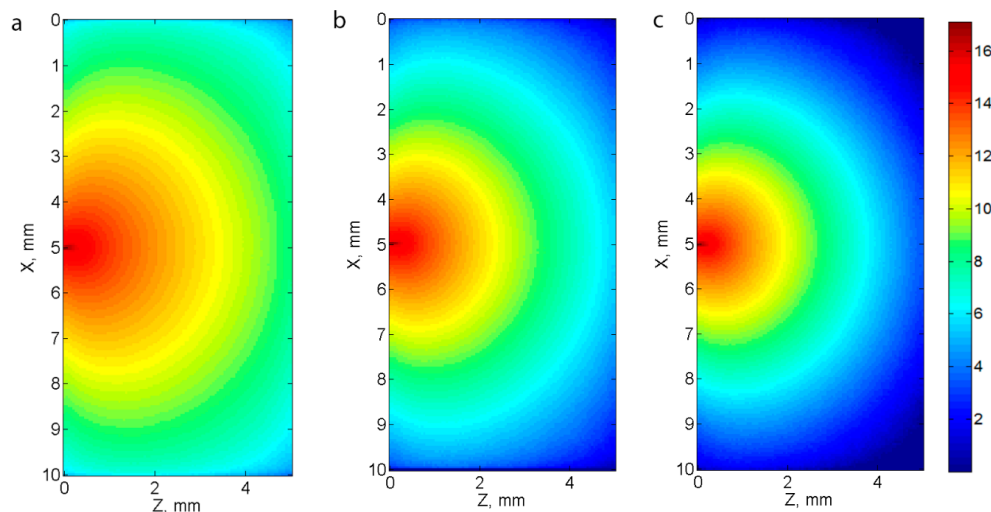


Fig. 1 Intensity maps for a collimated point source in tissues with $\mu_s = 20 \text{ mm}^{-1}$, $g = 0.8$ and different absorption coefficients: (a) $\mu_a = 0.088 \text{ mm}^{-1}$, (b) $\mu_a = 0.22 \text{ mm}^{-1}$, (c) $\mu_a = 0.35 \text{ mm}^{-1}$. Color encoding corresponds to the logarithm of intensity value.

first case the point source is located at the medium boundary and is directed normal to it. Detection of scattered light is performed by a wide-angle detector sized $0.05 \times 0.05 \text{ mm}^2$ located at the medium boundary at the distance in the range 0-10 mm from the source which covers the practical range for DOS applications.

Probing with structured illumination is simulated for two types of the incident light spatial modulation: i) one-dimension sinusoidal pattern with spatial frequency varying in the range of $0.3\text{--}2.5 \text{ mm}^{-1}$ and ii) one-dimension rectangular pattern with fixed width of stripes (0.1 mm) and duty cycles ranging between 0.03 and 0.25. Detection of photons was performed by the detector described above.

2.2 Monte Carlo technique

A custom developed MATLAB-based code adopted from our previous Monte Carlo solutions [14] was employed in the study. Heyney-Greenstein scattering phase function of the medium was applied. The code is optimized for MATLAB environment. Since MATLAB is efficient in arrays processing, the MATLAB realization of the Monte Carlo algorithm is based on the simultaneous processing of a definite number of photons chosen as 10^6 to ensure optimal PC performance and the use of RAM. The photon parameters are stored as arrays and are transformed in accordance with MC algorithm rules, i.e. 10^6 photons steps are processed at each iteration of the algorithm. At the stage of simulation initialization the initial parameters of each photon are generated (such as photon position, direction and weight) and stored in corresponding arrays. The photon trajectories are considered to be completed when the photon leaves the sample or the critically low weight is reached. This conditions are checked at each algorithm iteration and the parameters of photons satisfying these conditions are stored. The completed trajectories in the array are

replaced with the new ones initialized at the source position. The iterative process is performed until the number of completed photon trajectories reaches a predefined number equal to 10^7 in this study.

To model the effect of structured illumination the photon distribution over probing depths obtained for a point source was convolved with the considered illumination pattern resulting in the distribution corresponding to this pattern. In this calculations it was assumed that the overall probing power delivered to the object is the same for all the considered patterns.

2.3. Optical properties of studied tissue

Optical properties of the medium used in our MC simulations correspond to human skin at the wavelength of 600 nm [1,15] (see Table 1). In order to study the effect of optical properties on the distribution of photons over probing depth each of parameters was varied in the range of $\pm 60\%$ with respect to the basic values. The entire range of parameters is summarized in Table I. The refractive index of the medium amounted 1.4.

Table 1 Typical optical properties of human skin at the wavelength of 600 nm (basic value) and their variations employed in simulations (basic value - 60% / basic value / basic value + 60%).

μ_a, mm^{-1}	μ_s, mm^{-1}	g
0.088 / 0.22 / 0.35	8 / 20 / 32	0.8

3 Results and Discussion

3.1 Illumination with a collimated point source

Fig. 1 demonstrates typical simulated two-dimension maps of intensity scattered within tissue from a collimated point source for a definite scattering coefficient and different absorption coefficients. As

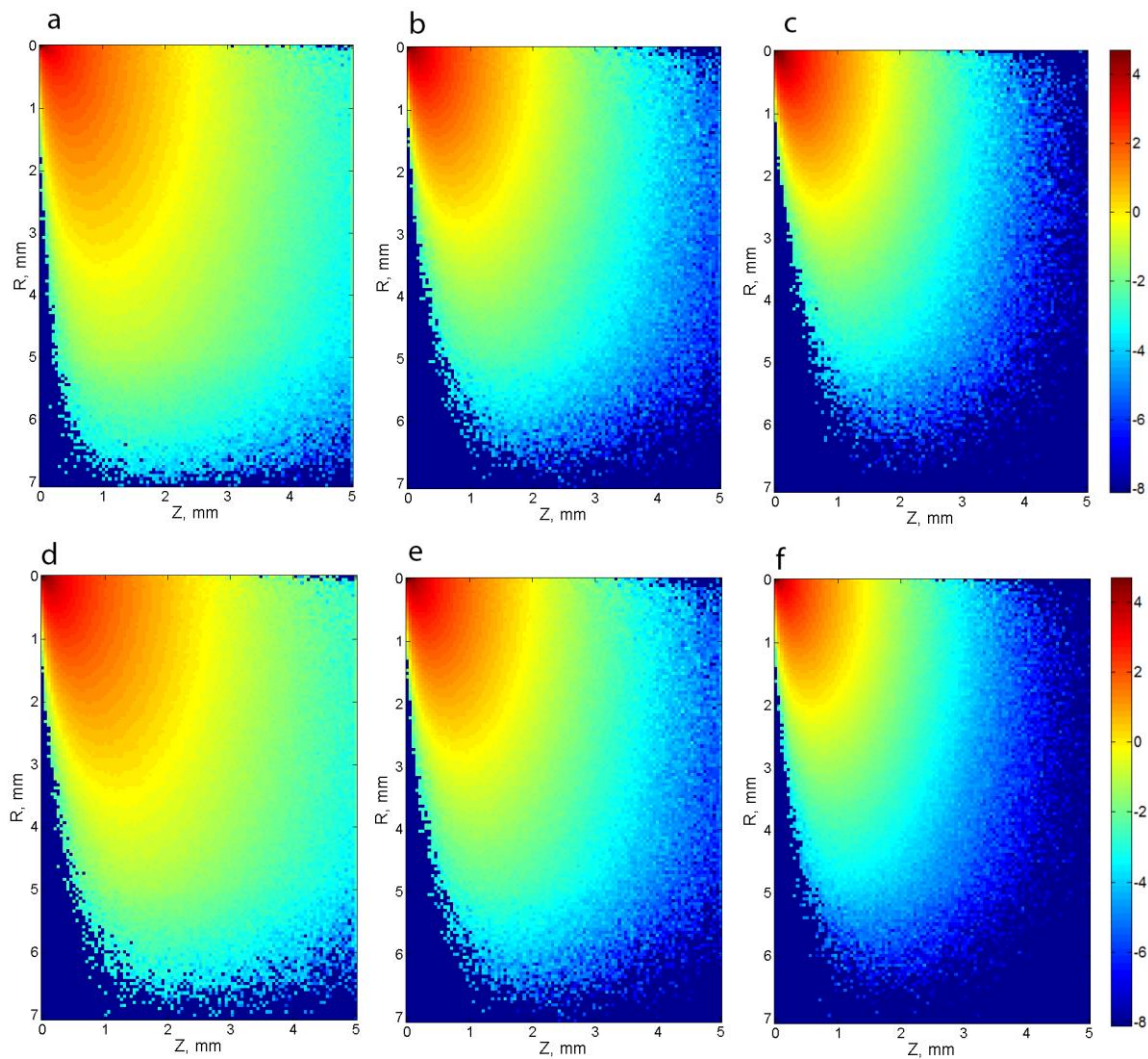


Fig. 2 Distribution maps of photons over probing depth Z reached in the medium (point source) for a source-detector separation R : (a) $\mu_s = 8 \text{ mm}^{-1}$, $\mu_a = 0.22 \text{ mm}^{-1}$; (b) $\mu_s = 20 \text{ mm}^{-1}$, $\mu_a = 0.22 \text{ mm}^{-1}$; (c) $\mu_s = 32 \text{ mm}^{-1}$, $\mu_a = 0.22 \text{ mm}^{-1}$; (d) $\mu_s = 20 \text{ mm}^{-1}$, $\mu_a = 0.088 \text{ mm}^{-1}$; (e) $\mu_s = 20 \text{ mm}^{-1}$, $\mu_a = 0.22 \text{ mm}^{-1}$; (f) $\mu_s = 20 \text{ mm}^{-1}$, $\mu_a = 0.35 \text{ mm}^{-1}$. Color encoding corresponds to the logarithm of the respective fraction of photons.

expected, stronger attenuation of intensity with the distance from the source corresponds to the case of higher absorption coefficient.

Typical distributions of photon trajectories contributing to reflectance signal over maximal depth reached in the medium (probing depth) are demonstrated in Fig. 2 as color-encoded maps where abscissa corresponds to probing depth, ordinate corresponds to source-detector separation and the logarithm of the respective fraction of photons is encoded with color. The results are demonstrated for the set of basic optical properties and for cases when the values of scattering and absorption coefficient are varied by $\pm 60\%$. The maps demonstrate that at small source-detector separation (R) the contribution of photons from the smaller depth (up to 0.2 mm) prevails while when the source-detector separation increases the most probable probing depth also increases reaching about 1 mm for $R = 5 \text{ mm}$.

With the increase of scattering coefficient (from Fig. 2a to Fig. 2c) the distribution shrinks to the region

of smaller probing depths and smaller source-detector separations. Higher scattering coefficient with all other parameters preserved results in longer photon pathways required to reach particular depth (due to multiple scattering) and, hence, stronger absorption at lower depth. Similar tendency can be observed for increase of absorption coefficient (from Fig. 2d to Fig. 2f): higher absorption results in shrinkage to lower probing depths and smaller source-detector separations. Similar response of the studied distribution to the variation of scattering and absorption coefficient can be explained by prevailing of diffusive regime of light propagation in the considered media. In diffusive regime the light propagation is governed by the effective attenuation coefficient

$\mu_{eff} = \sqrt{\mu_a(\mu_a + \mu_s(1 - g))}$; given $\mu_a \ll \mu_s(1 - g)$ the effect of variation of μ_s and μ_a becomes equivalent which is confirmed by the obtained results.

In order to study quantitatively the effect of optical properties on the photon distribution over probing

depth, the cross-sections of the corresponding distributions were plotted separately for different source-detector separations. Fig. 3 demonstrates the distributions of photon fractions over probing depth for several source-detector separations and for varying scattering coefficient. Distributions for $R = 0$ demonstrate monotonous decrease of the dependence on Z while for $R = 1$ mm and $R = 2$ mm the dependence demonstrates pronounced maximum. This maximum can be associated with the centers of the banana patterns [3] indicating DOS measurement volume. With the increase of R this maximum is shifted to the region of larger probing depths with simultaneous decrease of the fraction of contributing photons.

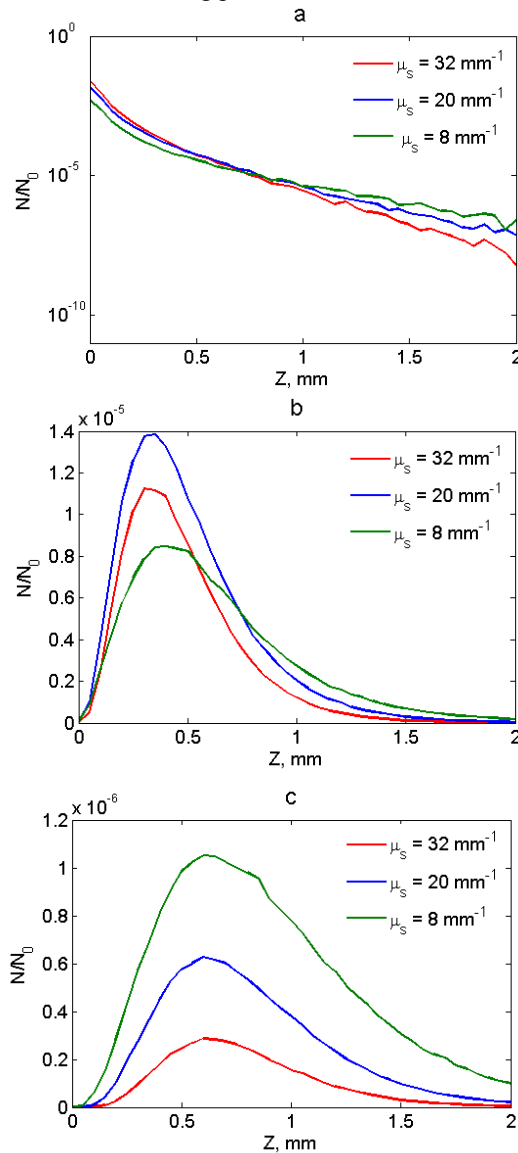


Fig. 3 Distribution of photons over probing depth for $\mu_a = 0.22$ mm⁻¹, $g = 0.8$ and different μ_s (shown in mm⁻¹) for $R = 0$ (a, semilogarithmic scale), 1 (b) and 2 (c) mm.

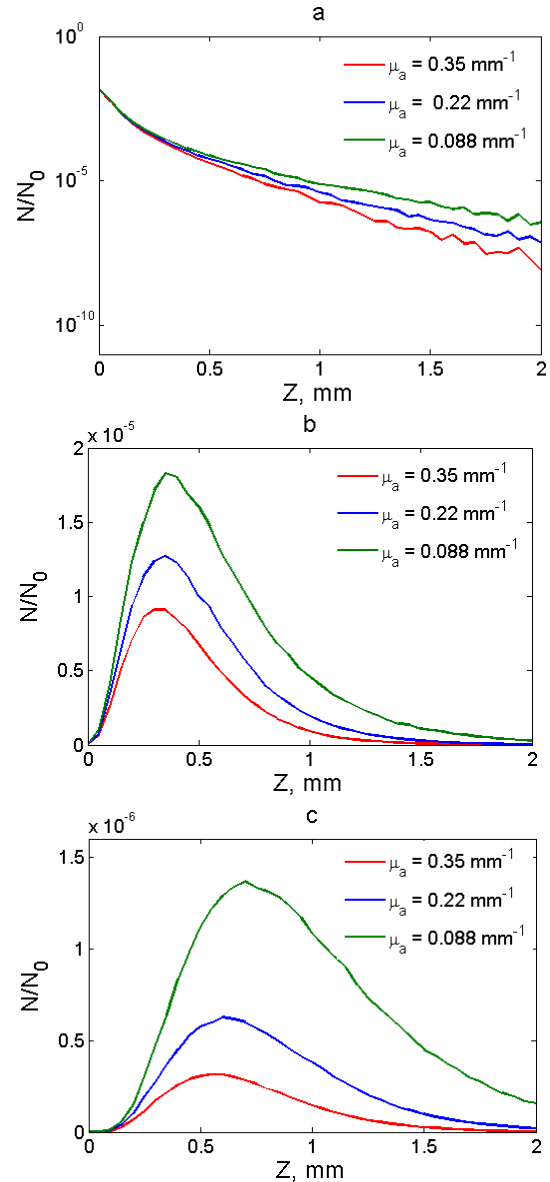


Fig. 4 Distribution of photons over probing depth for $\mu_s = 20$ mm⁻¹, $g = 0.8$ and different μ_a (shown in mm⁻¹) for $R = 0$ (a, semilogarithmic scale), 1 (b) and 2 (c) mm.

For $R = 0$ (Fig. 3a) the fraction of photons with small probing depth (below 0.8 mm) attenuates with μ_s decrease while the fraction of photons with higher probing depth (above 0.8 mm) increases. This fact is explained by the overall weaker attenuation of probing radiation with depth for smaller values of μ_s . For $R = 1$ mm the fraction of photons in the vicinity of the distribution peak is the highest for the basic parameters compared to higher and lower μ_s values (Fig. 3b). This fact is explained by two controversial effects: on the one hand, higher μ_s ensures higher scattering and, hence, contribution to backscattering signal; on the other, longer photons travel paths in medium results in higher absorption and, hence, lower photon fractions. As for the most probable probing depth, it is about 0.35 mm and. For $R = 2$ mm the effect of intensity attenuation due to absorption at longer pathlengths start

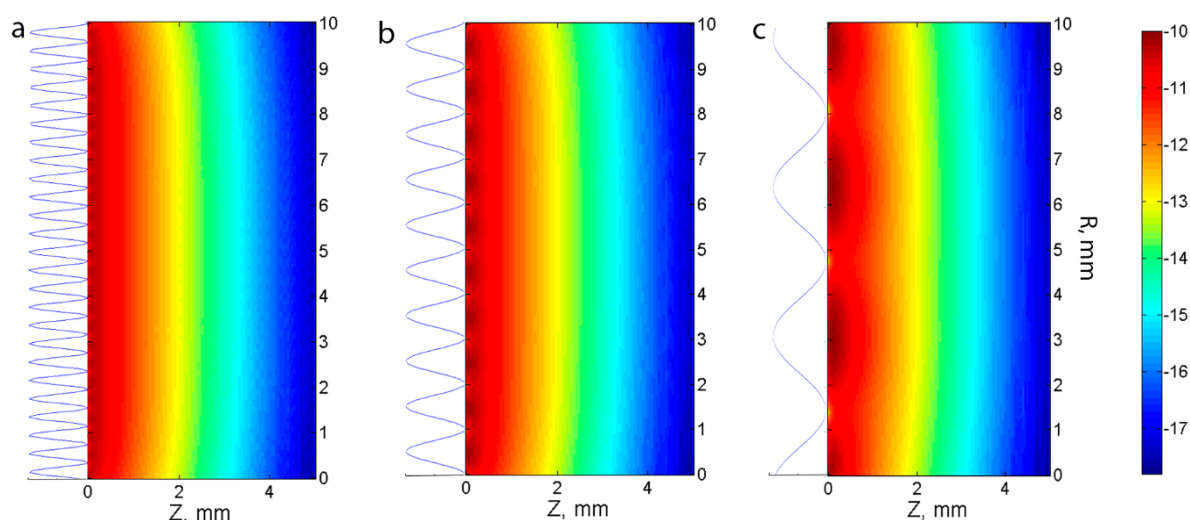


Fig. 5 Distribution maps of photons over probing depth for sinusoidal illumination pattern with different spatial frequencies f : (a) $f = 2.5 \text{ mm}^{-1}$; (b) $f = 1 \text{ mm}^{-1}$; (c) $f = 0.3 \text{ mm}^{-1}$. The profile of the illumination pattern is shown at the left. Color encoding corresponds to the logarithm of the respective fraction of collected photons.

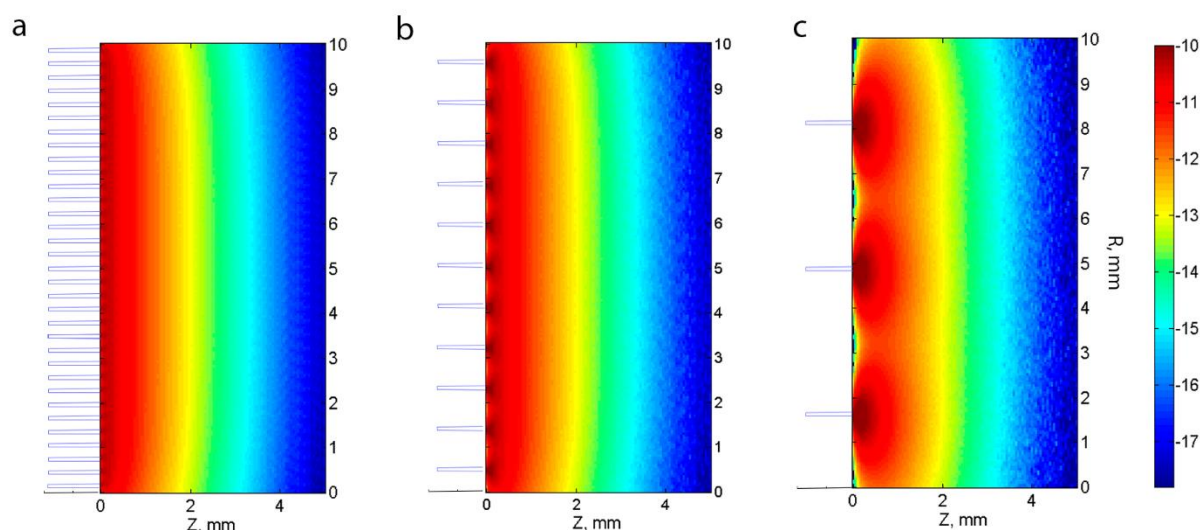


Fig. 6 Distribution maps of photons over probing depth for rectangular patterns with fixed width of stripes (0.1 mm) and various spatial duty cycles D : (a) $D = 0.25$; (b) $D = 0.1$; (c) $D = 0.03$. The profile of the illumination pattern is shown at the left. Color encoding corresponds to the logarithm of the respective fraction of collected photons.

to prevail and the amount of collected photons decreases with the increase in μ_s (Fig. 3c). The most probable probing depth for this R is about 0.6 mm.

On the contrary to variation in μ_s , variation in μ_a results in monotonous dependencies: the fraction of collected photons rises with the decrease in μ_a for all three considered R values (Fig. 4a). For $R = 1$ and 2 mm the most probable probing depth shifts to higher values with the decrease in μ_a (Figs. 4b and 4c). Overall, the most probable probing depth demonstrates stronger dependence on μ_a compared to μ_s .

3.2 Illumination with spatially modulated light

One of the modifications of DOS employs illumination with structured patterns of light in order to control measurement volume. Different parameters of the

pattern provide different distribution of probing radiation within the object. In this study we consider one-dimension sinusoidal and rectangular spatial patterns which are usually employed in measurements DOS systems. Sinusoidal patterns are more common [12,13] since they allow for analytical solution of diffusion equation with further ability to reconstruct a map of absorption coefficient [12]. It is assumed that probing volume significantly depends on the spatial frequency of the sinusoidal pattern employed.

Typical distributions of the detected photons over probing depth (abscissa) and the position of the detector (ordinate) for sinusoidal patterns with different spatial frequency are shown in Fig. 5. From this figure one can observe that for smaller probing depths (below 1 mm) the distribution dependence on R resembles the illumination pattern while for higher depths the

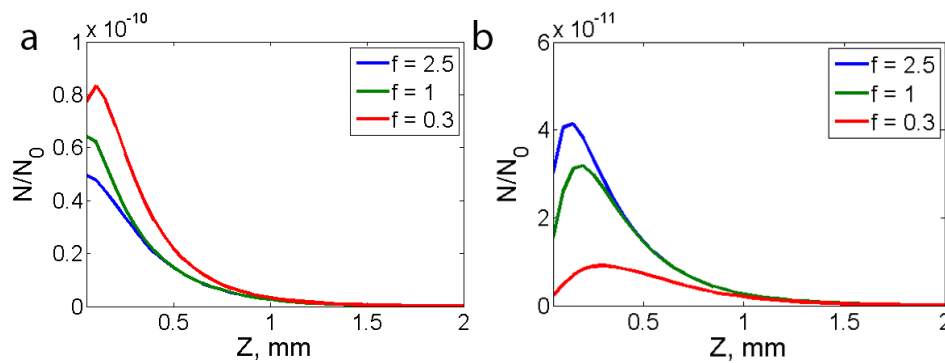


Fig. 7 Distribution of photons over probing depth for sinusoidal illumination patterns with different spatial frequency f for the detector position (a) at the center of bright stripe and (b) at the center of dark stripe.

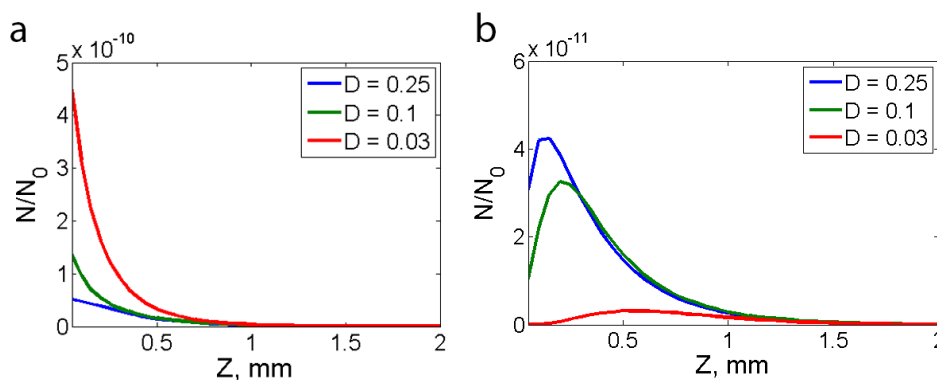


Fig. 8 Distribution of photons over probing depth for rectangular illumination patterns with different duty cycles D for the detector position (a) at the center of bright stripe and (b) at the center of dark stripe.

distribution remains almost uniform and unchanged for different spatial frequencies. The major changes are manifested when the detector is located at the minima of illumination patterns where the region of most probable probing depths shifts to higher values.

Similar trend was obtained for the case of rectangular patterns with the decrease in spatial duty cycle (Fig. 6), however, in this case the change in the structure of the probing depth map is more significant. This indicates higher potential of employing rectangular patterns for controlling measurement volume compared to sinusoidal patterns. Cross-sections of photon distributions over probing depth were plotted for transversal coordinates of the detector corresponding to centers of bright and dark stripes for both types of illumination pattern and are shown in Figs. 7 and 8, respectively. For sinusoidal patterns when the detector is placed at the center of bright stripe (Fig. 7a) the distribution maximum defining the most probable probing depth is located at the depth of about $Z = 0.1$ mm for spatial frequency $f = 0.3$ mm⁻¹ and shifts to the surface of biotissue sample with the increase of frequency. When the detector is placed at the center of dark stripe (Fig. 7b) the maximum of distribution is located at $Z = 0.25$ mm for $f = 0.3$ mm⁻¹ and, similarly, it shifts to the surface of biotissue sample with the frequency value increase. Note that the fraction of photons detected at the center of bright stripe is highest for $f = 0.3$ mm⁻¹ and decreases with the frequency

increase. On the contrary the fraction of photons detected at the center of the dark stripe increases with the frequency increase. This is explained by the fact that in the case of low spatial frequency local probing intensity is higher in the vicinity of the bright stripe center and is lower in the vicinity of the dark stripe center as compared to the case of higher frequency.

Similar results were obtained for rectangular illumination patterns (Fig. 8). The fraction of photons detected at the center of bright stripe is highest for the spatial duty cycle $D = 0.03$ and decreases with the duty cycle increase. On the contrary, at the center of dark stripe the fraction of collected photons increases with the duty cycle increase. As in case of the sinusoidal patterns, this fact originates from higher local probing intensity in the vicinity of bright stripe center compared to the dark stripe. In the case of rectangular pattern the difference is stronger which yields the shift of the distribution peak to the sample surface ($Z = 0$ mm) (Fig. 8a). When the detector is placed in the center of dark stripe, the position of the distribution peak demonstrates strong dependence on the duty cycle: the peak shifts from $Z = 0.6$ mm to $Z = 0.1$ mm when the duty cycle alters from 0.03 to 0.25.

4 Conclusions

In this work the effect of optical properties and probing pattern configuration on the probing depth of diffuse

optical spectroscopy is analyzed. Distributions of photons over maximal depth reached in the medium associated with probing depth are derived from numerical Monte Carlo simulations. For collimated point source it is shown that the fraction of collected photons demonstrates monotonous decrease with probing depth for source-detector separation equal to zero while with increase in source-detector separation a maximum at the definite depth is manifested. The position of this maximum shifts to higher probing depths with decrease of μ_a .

For the problem of structured illuminations the maps of photons distribution over probing depth are derived by the convolution of Monte Carlo results for a point source with the given illumination spatial patterns. Sinusoidal and rectangular patterns are considered. It is shown that the decrease in duty cycle and spatial frequency results in significant redistribution of the maps for rectangular and sinusoidal patterns, respectively. In particular, the distribution resembles illumination patterns for probing depths below 1 mm while for higher probing depths the distribution remains almost uniform and unchanged for different pattern parameters. Distribution of photons over probing depth demonstrates opposite trends when the detector is located in the center of bright versus dark stripe of the illumination pattern. For the center of bright stripe the peak of the discussed distribution remains close to the surface while for the center of dark stripe it shifts towards higher depths with decrease in spatial frequency and duty cycle for sinusoidal and rectangular patterns, respectively. Stronger dependence of this distribution on the duty cycle for rectangular patterns indicates high potential of this type of illumination pattern for controlling probing depth in DOS applications.

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Laser Illumination in Live Cell Microscopy: Scattering and Structured Illumination

Herbert Schneckenburger*, Verena Richter, Mathis Piper, and Michael Wagner

Aalen University, Institute of Applied Research, Beethovenstr. 1, 73430 Aalen, Germany

* e-mail: Herbert.schneckenburger@hs-aalen.de

Abstract. Two types of laser illumination in live cell microscopy with a focus on the sample or in the aperture plane of the microscope objective lens are distinguished. For the second case two examples are described, namely light scattering microscopy with angular resolution and Structured Illumination Microscopy (SIM) with two interfering laser beams. Appropriate applications include morphological studies of cells undergoing apoptosis and mitochondrial imaging with increased resolution. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Living cells; light scattering microscopy; super-resolution microscopy; structured illumination; SIM; apoptosis; mitochondrial imaging.

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1 Introduction

In epi-illumination microscopy, e.g. fluorescence or scattering microscopy, a light source is most commonly focused to the plane of a sample. If a laser beam is used for illumination, this creates a highly focused spot which may be used for laser scanning microscopy [1,2] or for laser micro-manipulation, e.g. in an optical tweezer system [3]. In some special illumination systems laser irradiation is separate from the microscope lens, i.e. it is perpendicular or occurs under a certain angle to the detection path. This kind of illumination has been realized in light sheet microscopy [4,5] or in prism based total internal reflection fluorescence microscopy (TIRFM) [6]. Comparably little attention has been paid to epi-illumination in a conventional microscope, where the laser beam is focused to the aperture plane of the objective lens which is generally placed in its rear focal plane. Depending on the exact position of the focus this results in a parallel beam in the plane of the sample under a well-defined angle of illumination including super-critical angles in objective-type TIRFM [6]. The present paper describes two setups and applications of this illumination technique, namely light scattering microscopy with angular resolution and Structured Illumination Microscopy (SIM) with two interfering laser beams.

2 Materials and Methods

2.1 Cells and samples

For experiments of light scattering microscopy 3T3 murine fibroblasts were grown as monolayers in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, pyruvate and antibiotics at 37°C and 5% CO₂. Apoptosis was induced by addition of 2 µM staurosporine for 4 hours, as reported earlier [7]. The occurrence of apoptosis under these conditions was verified previously using a Förster Resonance Energy Transfer (FRET) based sensor for caspase-3 [8]. For experiments of Structured Illumination Microscopy (SIM) we used small clusters of human MCF-7 breast cancer cells cultivated in Dulbecco's MEM/Ham F-12 at 37°C and 5% CO₂. Cells were incubated with the mitochondrial marker rhodamine 123 (R123; 5 µM) for 30 minutes prior to rinsing with Earle's Balanced Salt

Solution and fluorescence microscopy using a Plan Neofluar 40×/1.30 oil immersion objective lens together with a cover slide. Microscope slides and standards were used for additional test experiments.

2.2 Illumination

Fig. 1 shows the difference between focused illumination of a sample, used e.g. in laser scanning microscopy or an optical tweezer system (Fig. 1a), and focused illumination of the aperture plane (Fig. 1b). In the first case (Fig. 1a) a lens L_2 focuses the light source to a field diaphragm FD at the entrance of the microscope which is in a conjugated plane of the sample S. Then the tube lens L_3 of the microscope creates a parallel beam which passes the aperture A of the microscope objective lens L_4 and is finally focused on the sample S. In the second case (Fig. 1b) a parallel beam passes the field diaphragm (FD) and is focused by the tube lens L_3 to the aperture plane in the rear focus of the objective lens, thus generating a parallel beam in the plane of the sample. Assuming a beam diameter of about 15 mm in front of the tube lens L_3 (with a typical focal length of 164 mm), this results in a numerical aperture $A_N = 0.045$ and finally in a spot diameter $d = 1.22 \lambda / A_N \approx 13 \mu\text{m}$ in the aperture plane of the objective lens, thus permitting about 460 positions of the laser spot across a 6 mm aperture. Often telescopes consisting of the lenses L_1 and L_2 are used for illumination, but only in the second case the light beam is parallel at the entrance FD of the microscope, whereas in the first case a focus is created. A pinhole P within the telescope (i.e. between the lenses L_1 and L_2) may fulfill various purposes, e.g. filtering of laser modes or selection of well-defined diffraction orders from an optical grating.

Illumination with a focus in the aperture plane of the microscope objective lens according to Fig. 1b has been used for *scattering experiments with angular resolution* [7]. In this case the collimated (parallel) laser beam is deflected by a mirror under a variable angle prior to expansion by the telescope to a final diameter of about 15 mm. In the present microscope (Axiovert 200M, Carl Zeiss Microsystems GmbH, Jena, Germany) the tube lens is replaced by an assembly of three lenses without any loss of aperture and resolution, so that an angular range of about 100° can be covered by the microscope

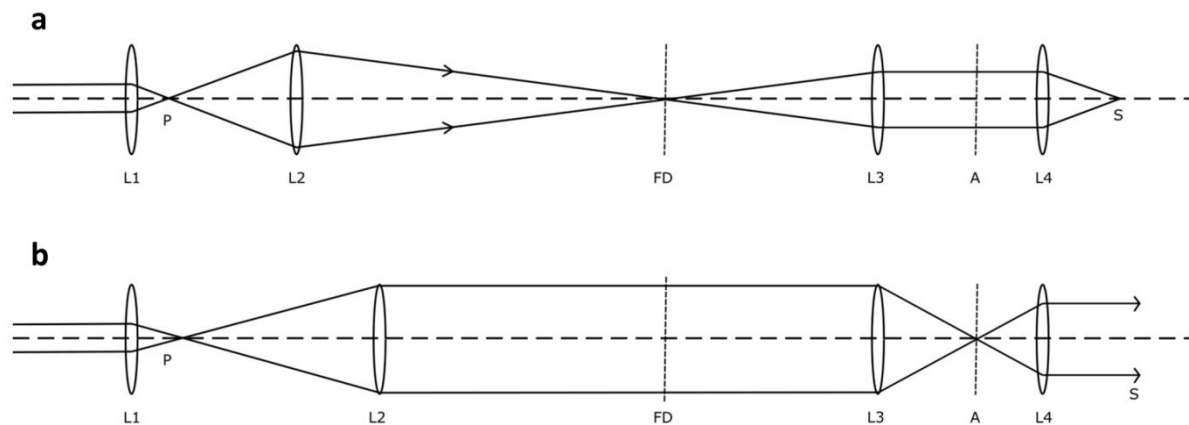


Fig. 1 Laser illumination in microscopy (a) focused on a sample S or (b) in the aperture plane A of the objective lens (lenses L_1 , L_2 , L_3 and L_4 , field diaphragm FD, aperture A).

with a resolution of $0.2\text{--}0.3^\circ$. Backscattered light can be collected by the same objective lens, collimated by a tube lens and focused by a so-called Bertrand lens to an exit pupil with a pinhole located in its centre, as depicted in Fig. 2. An angle of $180 \pm 1^\circ$ can thus be selected prior to light detection by a photomultiplier. Although backscattering can be measured in a large range of $130\text{--}230^\circ$ between the exciting and the scattered laser beam, an angular range of $190\text{--}220^\circ$ (or alternatively $140\text{--}170^\circ$) often revealed to be sufficient, so that specular reflection as well as large inclination angles (which might give some erroneous results) can be excluded.

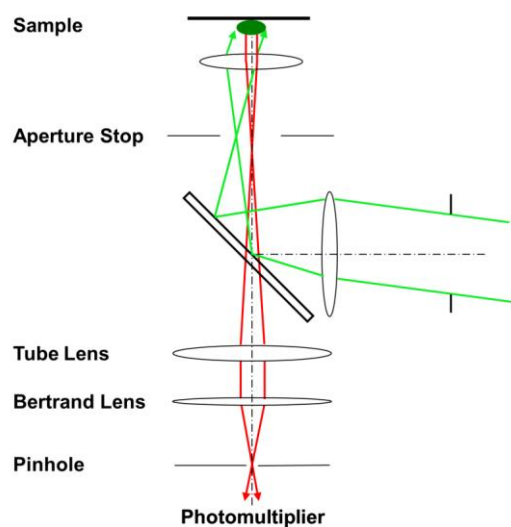


Fig. 2 Variable-angle light scattering microscopy. Entrance optics of the microscope may consist of a single tube lens (as indicated in the Fig.) or an assembly of several lenses (reproduced from [6] with modifications).

In *Structured Illumination Microscopy (SIM)* an interference pattern of at least two laser beams is created in the plane of the sample. In comparison with

conventional fluorescence microscopy this gives higher spatial frequencies, broadens the optical transfer function (OTF) and increases resolution up to a factor 2 [9,10]. Two laser beams (e.g. the first diffraction orders of an optical grating or a spectral light modulator (SLM) upon blocking of $k = 0$ [11]) create an interference pattern at the entrance plane FD of the microscope. They are further focused at the edges of the microscope aperture, as depicted in Fig. 3a, to get optimum resolution. In the present case an angle $\alpha = 1.02^\circ$ between the optical axis and each interfering laser beam was adjusted using a focal length $f' = 150\text{ mm}$ for the lens L_2 . This results in a grating constant of the interference pattern $d = \lambda / 2 \sin \alpha = 13.71\text{ }\mu\text{m}$ in the entrance plane FD and 343 nm in the plane of the sample at $40\times$ diminution (Fig. 3b) or 218 nm at $63\times$ diminution by the objective lens L_4 (in combination with the tube lens L_3). From 3 rotation angles of the optical grating (0° , 60° and 120°) and 3 phases of the interference pattern in each case (0 , $2\pi/3$, $4\pi/3$) a resulting unstructured image with enhanced resolution (up to a factor 2 in comparison with conventional microscopy) can be calculated after Fourier transformation [12]. It should be mentioned that a disk of 6 pinholes can be placed in the common focus of the lenses L_1 and L_2 to select the 1st diffraction orders of the optical grating or SLM, and that a $\lambda/4$ plate together with a rotatable polarizer can be further introduced in the illumination rays to get interference in a well-defined plane of polarization.

3 Applications

An example for *light scattering microscopy with angular resolution* is given in Fig. 4 for the case of apoptosis of 3T3 murine fibroblasts. Light scattering is depicted from a small object field of 4–6 individual cells prior to (control) and subsequent to apoptosis (induced by addition of $2\text{ }\mu\text{M}$ staurosporine to the culture medium for 4 hours). While the cells show pronounced changes in morphology after initiation of apoptosis (characterized by shrinking and almost

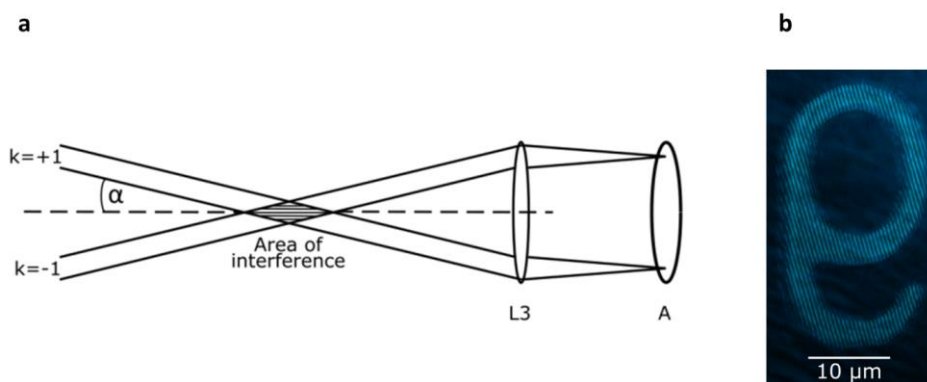


Fig. 3 (a) Interference of 2 laser beams used for Structured Illumination Microscopy (SIM) with laser foci in the aperture plane A; (b) Interference pattern on a microscope standard upon SIM by 2 interfering laser beams (Ar^+ laser, 488 nm) with a Plan Neofluar 40 $\times$ /1.30 oil immersion objective lens using a modified Axiovert 200M microscope (Carl Zeiss).

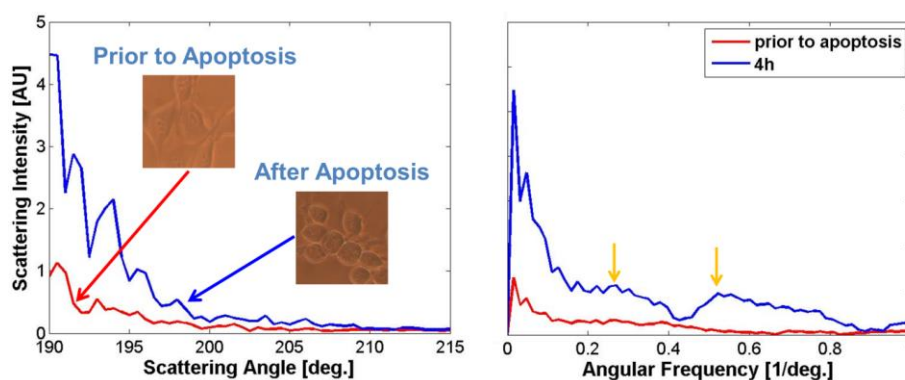


Fig. 4 Scattering intensity of 3T3 fibroblasts prior and subsequent to apoptosis (induced by 2 μM staurosporine after 4h); angular resolution (left) and Fourier transform (right); scattering maxima are indicated by arrows.

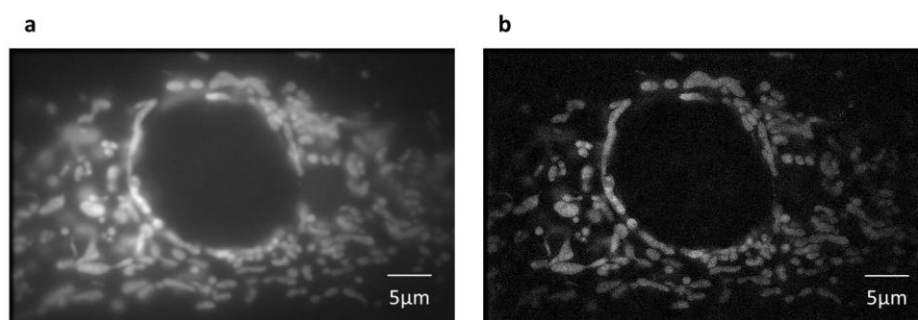


Fig. 5 MCF-7 breast cancer cells incubated with the mitochondrial marker R123 (5 μM , 30 min.) excited by 2 interfering rays of an Ar^+ laser (excitation wavelength: $\lambda_{\text{ex}} = 488 \text{ nm}$; detection range: $\lambda_{\text{D}} \geq 515 \text{ nm}$; Plan Neofluar 40 $\times$ /1.30 oil immersion microscope objective lens); (a) wide field image; (b) SIM image calculated from 9 structured images.

spherical shape, see insets of the Fig. 4a), the scattering intensity increases and exhibits pronounced oscillations, as described in the literature for Mie scattering [13]. After Fourier transformation maxima of the scattering function are resolved at angular frequencies close to 0 as well as around 0.3 and 0.5 oscillations per degree (Fig. 4b). A similar behaviour resulted from more than

15 other cell clusters (of 4 to 6 cells each) as well as a smaller number (≤ 10) of multicellular spheroids [7]. In an attempt to describe this scattering behaviour, Mie scattering was simulated for a sphere of variable diameter and a refractive index $n_{\text{C}} = 1.38$ (cell) surrounded by a medium of refractive index 1.33, as well as for a sphere with a concentric inclusion of

variable diameter and a refractive index $n_N = 1.42$ (cell nucleus). Best results were obtained for a cell of $12.4\ \mu\text{m}$ diameter with a nucleus of $9.3\ \mu\text{m}$, which fairly corresponds to visual observation.

Fluorescence images of individual MCF-7 breast cancer cells incubated with the mitochondrial marker rhodamine 123 (R123; $5\ \mu\text{M}$, 30 min.) are depicted in Fig. 5. In both parts of this image a non-fluorescent nucleus is surrounded by mitochondria which appear as long-shaped fluorescent rods. While the wide-field image (Fig. 5a) shows some overlaying diffuse out-of-focus fluorescence, the image calculated from 9 images with *structured illumination* (SIM; Fig. 5b) shows fluorescence only from the focal plane with improved resolution.

4 Discussion

Laser illumination with a focus in the rear focal plane of a microscope objective lens permits various applications with high angular resolution. In this paper we presented elastic light scattering as a method for detection of cell morphology, which may be suitable for studies of apoptosis as well as for measurement of infiltration of tumor cells into a host tissue [7]. In addition, angular resolution appears to be appropriate for objective-type Total Internal Reflection Fluorescence Microscopy (TIRFM) [6] or for dark field illumination in Raman microscopy.

Focusing of laser light in the rear focal plane of a microscope is also an appropriate method for interference microscopy, e.g. for creating an interference pattern in the plane of a sample which might be used for Structured Illumination Microscopy (SIM) with increased resolution. Lateral resolution cannot be determined quantitatively by the present experiments, but can be estimated from preliminary results of reference samples (fluorescent beads) and calculated from the sum of spatial frequencies $k_A = 2A_N / \lambda$ according to the Abbe criterion (with the numerical aperture A_N and the fluorescence wavelength λ) and $k_G = 1/d$ of the optical grating (with the grating constant d in the plane of the sample) [9]. In our case with $A_N = 1.30$, $\lambda = 520\ \text{nm}$ and $d = 343\ \text{nm}$ (with a $40\times$ objective lens) we obtain $k = k_A + k_G = 0.0079\ \text{nm}^{-1}$ corresponding to a resolution $1/k = 126\ \text{nm}$. A $63\times$ objective lens would result in a grating constant $d = 218\ \text{nm}$, a spatial frequency $k = 0.0096\ \text{nm}^{-1}$ and a resolution of $104\ \text{nm}$ which is by about a factor 2 better than the resolution resulting (only) from the Abbe criterion. Furthermore, in axial tomography [14] a sample can be rotated by variable angles, so that this very high resolution can be obtained in any direction of the sample. Presently a series of 9 structured images can be recorded with a light dose of about $10\ \text{nJ}/\mu\text{m}^2$ corresponding to the light dose after 10 seconds of solar exposure. This light dose is more than 1000 times lower than corresponding values obtained with other methods of super-resolution microscopy (e.g. STED [15], PALM [16], STORM [17] and related methods [18]) and allows

living cells to be examined under non-phototoxic conditions [19] repeatedly over a longer time period or under variable angles of illumination.

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Spectral Analysis of Nanodiamond-Berberine Complex Interaction with Living Cells for Nanoparticle Mediated Drug Delivery

Yu-Chung Lin^{1,2}, Zhe-Rui Lin¹, Lin-Wei Tsai¹, Elena Perevedentseva^{1,3}, Artashes Karmenyan¹, and Chia-Liang Cheng^{1*}

¹ Department of Physics, National Dong Hwa University, Hualien, Taiwan

² Institute of Physics, Academia Sinica, Taipei, Taiwan

³ P. N. Lebedev Physics Institute of Russian Academy of Science, Moscow, Russia

* e-mail: clcheng@gms.ndhu.edu.tw

Abstract. The natural isoquinoline alkaloid berberine has been demonstrated for its significant activity against a variety of diseases caused by bacterial and parasite infections. Nanodiamond (ND) has also been proven to have great potential for drug delivery. In this work, the complex of ND with berberine is constructed through surface functionalized ND, aiming for drug delivery applications. Spectroscopic analysis was performed on the complex interaction with human lung alveolar carcinoma epithelial cell (A549) and red blood cell (RBC) in comparison with berberine in solution. The studied cells were treated with carboxylated ND, ND-berberine complex and berberine, respectively. Their distributions in cells structures are visualized using ND and berberine's fluorescence signals for detection. The distribution of ND and ND-berberine complex differs with the distribution of berberine introduced in solution form. The results show that the ND with attached drug molecules can act as a drug carrier allowing controllable localization of the drug into the cell. This result can have implication in drug delivery, release and delivery tracing applications. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Nanodiamond; Berberine; Bio-Applications; Medical Applications; Fluorescence; Surface Functionality; Imaging; Drug delivery; Nanobiotechnology.

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1 Introduction

Nanodiamond (ND) is recently considered to be a promising material for various bio-medical applications due to its unique features in sizes, structure, surface chemistry, physical properties and biocompatibility [1, 2]. Particularly, the optical-spectroscopic properties of ND such as Raman and fluorescence [3, 4] open wide possible applications for bio-imaging and bio-sensing [5]. Its surface structure allows forming complex of ND with molecules of interests (drugs, antibodies, genes, etc.) [6, 7], and used as carrier for delivery of these molecules [8, 9] or the combination of delivery and imaging for monitoring and tracing [10].

Develop methods of delivery using ND implies analysis of functionality of bio-active molecules attached on ND [11, 12] and studies of mechanism of ND internalization by cells. It has been shown that nanodiamond of various sizes penetrates into different cells via clathrin-mediated endocytosis and localized in the cytoplasm [13, 14]. Nanodiamond's properties and surface modification with bio-active molecules can alter the ND penetration into cell [15]; this effect can be used to control the internalization of ND in cells. Functionality of proteins attached on ND (e.g. enzyme [11], hemoglobin [16, 17], lipoproteins [12], different drugs [8-10, 18-20]) has been investigated. Difference in mechanism of interaction of nanodiamond conjugates and pure drug with the target objects can modify the treatment [18], particularly, to overcome the chemoresistance of tumor [19, 20]. Thus, using ND based conjugates allows improving efficiency of the treatment and decreasing the possible side effects.

It is obvious that optimal use of these advantages requires thorough studies of ND conjugates interaction mechanism with targeted systems. In the present work, we compare the internalization effects in cell of nanodiamond conjugate and pure drug. Nanodiamond-berberine complex is prepared to study the interaction of nanodiamond-drug complex with cells using plant alkaloid berberine for the conjugation. Berberine, a clinically important natural isoquinoline alkaloid, has been extensively studied for its multiple biological activities, particularly its activity against bacteria, fungi and viruses [21]. A wide range of other pharmacological effects, including anti-inflammatory [22] and anti-cancer [23] effects, etc. [24] have also been reported. Recently the research interest on berberine is growing dramatically, large number of investigations show efficiency of the berberine in diabetes and metabolic syndrome treatments, improving glucose and lipid metabolism disorders [25, 26]. Berberine targets a very basic regulator of metabolism adenosine monophosphate-activated protein kinase (AMPK), regulating energy homeostasis. Its action is based on increasing AMPK activity [27, 28].

Berberine exhibits well-detectable fluorescence, and some attempts to utilize this fluorescence were made, particularly for cellular imaging and diagnostics [29] and bio-sensing via fluorescence resonance energy transfer (FRET) [30]. Berberine fluorescence is sensitive to and can be modified by an environment chemical composition and properties [31]. It also has been reported as a photosensitive agent, which is able to produce singlet oxygen and radical species under UV irradiation [32]. The preparation of ND-berberine complex and analysis of delivery and release as well as chemo- and photosensitivity are interesting itself. In the present study berberine is used as a drug with bright fluorescence allowing its detection in the cell. In this study the red blood cells (human RBC) and human lung alveolar carcinoma epithelial (A549) cells are treated with ND-berberine complex, berberine in solution and ND. Their relative distributions in cellular structures are observed using ND and berberine's fluorescence signals for detection and analyzed to determine the difference from point of view of control of delivery.

2 Methods and Materials

2.1 Nanodiamond and Nanodiamond-berberine complex preparation

Nanodiamond and berberine complex was prepared using ND with nominal size 100 nm (Kay Diamond, US). Detail method of ND treatment has been reported elsewhere [33]. In short, nanodiamond was treated with mixture of strong acids $\text{H}_2\text{SO}_4:\text{HNO}_3$ (1:3), to remove non-diamond admixtures and contaminations, and to modify the particles with COOH surface functional groups (carboxylated nanodiamond, cND) for further conjugation with desired molecules. Throughout the text, all the ND has been acid treated. For simplicity, in this work, ND means carboxylated nanodiamond.

Berberine (2, 3-methylenedioxy-9, 10-dimethoxyprotoberberine Chloride) was obtained from Sigma-Aldrich (USA). To prepare the complex, 4 mg of berberine powder was first dissolved in 1 ml of standard phosphate buffer saline (PBS: NaCl 4 g; KCl 0.1 g, Na_2HPO_4 0.72 g, KH_2PO_4 0.21 g, H_2O 500 ml; pH 7.4) and heated to 40 °C for 30 min to increase solubility. Then, 2 mg/ml of ND was added to the berberine solution, obtaining its suspension with concentration of 2 mg/ml. The mixture was thoroughly agitated for 2 hrs for better adsorption of the berberine. After the agitation the mixture was centrifuged at the speed of 11,000 rcf at room temperature for 15 minutes to sediment the nanodiamond, including ND with adsorbed berberine. Then, 1 ml of PBS solution (pH value is 7.4) was added into the sediment, containing ND-berberine complex. The complex was subjected to weak ultrasound

treatment to disaggregate the sediment and then to vortex for 30 minutes. The washing of ND-berberine complex from solution to remove non-interacting berberine was repeated 3 times.

2.2 UV-Visible absorption measurements

The absorption spectra of berberine solution before and after interaction with ND were measured using UV-Visible spectrometer JASCO V-550 (JASCO, US) at room temperature. The intensity of adsorption peaks of the berberine are proportional to concentrations of the berberine in the solution, so comparison of the spectra allows rough estimation of quantity of adsorbed berberine and strength of the adsorption. In short, the spectrum of the prepared solution was measured to obtain the initial concentration of the berberine. Then, ND is added to adsorb the berberine. After centrifugation, the supernatant is measured. The difference the berberine adsorbed on the ND. The sediment is then washed with deionized water and centrifuged to ensure the sediment is ND-berberine, not ND and berberine mixture. The progress is repeated until we have zero absorption on the measured spectrum on the supernatant.

2.3 Size and ζ -potential measurements

The particle size and ζ -potential were analyzed using the Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) with a 4 mW, 633 nm wavelength He-Ne laser on the base of dynamic light scattering method, with the detection angle 173°. Nanodiamond, berberine and ND-berberine complex were diluted with PBS to measure size and surface charge to obtain the concentrations. After dilution to 20 $\mu\text{g/ml}$, the pH was 7.4 at 25 °C. The pH values were measured using a SENTRON pH-meter (Titan, Taiwan).

2.4 FTIR, Fluorescence and Raman spectra measurements

For FTIR, fluorescence and Raman spectroscopic characterization, 20 μl of ND, berberine or ND-berberine complex solution each was dropped on a Si substrate (1 cm $\times$ 1 cm) and dried in air under room temperature. FTIR spectroscopy (ABB Bomem MB 154 FTIR spectrometer, Switzerland) with a Deuterated Tri-Glycine Sulfate (DTGS) detector was used to obtain the sample's infrared spectra to confirm the forming of ND, berberine and ND-berberine complex respectively at temperature 25 °C in air. The spectral resolution was 4 cm^{-1} . The Raman and fluorescence spectra were measured using Raman spectrometer (T64000, Jobin Yvon, France) with 633 nm (for Raman spectra) and 325 nm (for fluorescence spectra) excitation wavelength.

2.5 Red Blood Cells (RBC) solution preparation

Fresh human blood was obtained from healthy volunteers and was transferred into the EDTA-covered tube; the research methods were approved by the Research Ethics and Use Committee of Buddhist Tzu Chi General Hospital (approval REC No. IRB101-149). The RBC were separated from the blood using centrifugation with the speed 1500 rcf for 5 min at 4 °C (HERMLE Z 323 K, Germany) and then repeatedly (5 times) washed with standard PBS by dissolving in ratio 1:2 and centrifugation with speed 3400 rcf at 4 °C for 1 minute. Finally, the washed RBC was diluted with PBS in the ratio of 5 μl : 1000 μl (RBC: PBS).

To study the interaction of 100 nm ND, berberine and ND-berberine solutions with RBC, ND and ND-berberine were suspended in PBS with concentration 10 mg/ml. 20 μl of the ND+PBS suspension was mixed with 1500 μl of PBS and added to 1005 μl of RBC+PBS suspension. Final concentration of ND or ND-berberine in the prepared ND (or ND-berberine) +RBC+PBS sample used for the measurements was $\sim$ 20 $\mu\text{g/ml}$. For the berberine interaction with RBC, the berberine was dissolved in PBS in concentration of 4 mg/ml. The berberine solution (100 μl) was mixed with 1980 μl of PBS and added to 10 μl of RBC suspension. The final concentration of berberine in the prepared berberine+RBC+PBS sample used for the measurements was $\sim$ 200 $\mu\text{g/ml}$.

2.6 A549 cell culturing

Human lung alveolar carcinoma epithelial cell A549 was obtained from Bioresource Collection and Research Center (BCRC) in Taiwan. A549 cells were cultured in RPMI1640 medium (Gibco, Invitrogen, UK). The medium was supplemented with 2 mM L-glutamine (Invitrogen, USA), 1.5 g/L sodium bicarbonate (Sigma, UK), 10% fetal bovine serum (Gibco/Life Technologies, Carlsbad, CA, USA). Cells were maintained under standard cell culture conditions in an incubator (Galaxy 170S, Eppendorf, USA) containing 95% air and 5% CO₂ at 37 °C humid environment. Culture medium was replaced with fresh medium every 48 or 72 hr. Cells were detached by treatment with 0.5% trypsin and 2.6 mM ethyl-enediaminetetraacetic acid (EDTA) (Gibco/Life Technologies, Carlsbad, CA, USA); cultures were subcultured routinely at approximately 80% confluence.

The A549 was treated with ND, berberine and ND-berberine to observe their interaction. Each sample was added to the medium, the sample concentration in the medium was 20 $\mu\text{g/ml}$, and cells were incubated together with the samples for 2 hours. Unreacted samples were removed by washing. The cells with ND, berberine and ND-berberine adhered on the coverslips were used for microscopic investigations.

2.7 Cells Raman measurements and confocal fluorescence imaging

Raman spectroscopic analysis of RBC was performed according to methods described previously [16, 17]. For Raman spectroscopic investigation of ND-berberine and berberine interaction with RBC, the Raman spectra of RBC determined by hemoglobin (Hb) were measured using a Raman spectrometer (T64000, Jobin Yvon, France) with 633 nm excitation wavelength laser. The prepared RBC+PBS and the ND-berberine+RBC suspensions were placed in a Petri dish of 5 cm diameter; the Si substrate 2×2 cm² was kept at the bottom during the initial microscopic measurements to avoid the interference of the signals from the plastic Petri dish. The oxygenation/deoxygenation of ND-berberine interacted RBC can be observed via the changes of a single RBC's Raman spectra. Oxygenation was achieved in ambient atmospheric pressure; deoxygenation was performed with nitrogen gas (10 kg/cm²) purged into the Petri dish. RBC spectra were measured for one cycle of deoxygenation-oxygenation for a single RBC, and each experiment was repeated with 5-10 RBCs.

Imaging of the cells (RBC and A549) after interaction with berberine, ND and ND-berberine complex was performed using a Leica laser-scanning confocal fluorescence microscope (TCS SP5, Mannheim, Germany) with 40× 1.25 oil immersion objective (Leica, Mannheim, Germany). To localize ND and berberine relatively the cell structures the confocal scan along vertical direction (Z-scan) was applied. The berberine fluorescence was excited with 405 nm wavelength laser and signal was collected in the 530-620 nm wavelength spectral range; ND's fluorescence was excited with 488 nm wavelength laser and signal was collected in the 500-530 nm wavelength range.

3 Results and Discussion

The characteristic UV-Visible absorption spectra of berberine are shown in Fig. 1. The absorption spectrum reveals three strong bands with maxima around 250 and 350 nm, which are characterized as $\pi \rightarrow \pi^*$ transition [34], and forth relatively weak band centered at about 430 nm. In Fig. 1, spectrum (I) corresponds to absorption of the initial solution of the berberine prepared. Spectrum (II) is the supernatant after first centrifugation. The difference in the solutions spectra intensities is proportional to quantity of the substance (in this case berberine) which was removed from the solution in the result of the interaction with nanodiamond [33]. Spectra (III)-(VI) display spectra of supernatant after following washings of nanodiamond sediment. One can see that significant quantity of the berberine is washed out from the nanodiamond, especially at 2nd washing (spectrum III). However the measurements discussed below show that the quantity of the berberine in ND-berberine complex is enough for observation and for further study of the complex interaction with cells.

The measured Raman and FTIR spectra of the berberine are presented in Fig. 2 after baseline correction. The vibrational spectra of berberine and some derivatives have been investigated previously using the Raman (including SERS) and IR absorption spectroscopy methods [35-36]. Both, Raman and FTIR spectra are complicate and the most intense peaks are positioned in the range 1200-1700 cm⁻¹. The vibrational spectrum of berberine reveals CH bending and aromatic CC stretching modes, together with CO stretching vibrations, CH bending in the OCH₂ and OCH₃ groups, and aliphatic CH bending, corresponding to detailed attribution given in the literature [36]. Because the spectra are measured in ambient conditions, the OH vibrations around 1620-1650 cm⁻¹ overlap with the sample spectra.

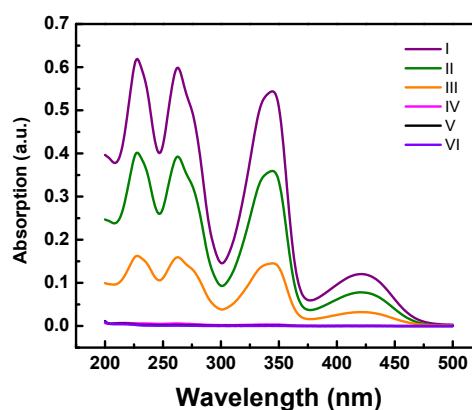


Fig. 1 UV-Visible spectra of berberine. (I) Initial solution of the berberine, (II) Supernatant after first centrifugation, (III)-(VI) Supernatant after following washing of nanodiamond sediment.

Raman spectrum of 100 nm ND reveals diamond peak of sp³ hybridized carbon at 1333 cm⁻¹. The weak but observable diamond peak in the spectrum of ND-berberine complex confirms the berberine adsorption on ND. While the agreement between ours, literature experimental data, and calculations [35-36] for the berberine Raman spectra are very good. However, for IR all measured spectra differ from calculated spectra significantly. This is because the dominant bands of the berberine are due to vibrations of highly polarized bonds and are therefore very sensitive to the experimental conditions (particularly, aggregation state and H-bonding) [35-36].

In Fig. 3, the sizes (a) and ζ -potentials (b) of ND, berberine, ND-berberine complexes obtained using dynamic light scattering method measured at pH~7.45 are presented. The size of ND particles is close to nominal size 100 nm (155±20 nm), so the degree of aggregation is low and well dispersed ND solution was achieved. In the case of ND-berberine complex significant fraction of non-aggregated particles (ND-berberine complexes) with average size about 170 nm also exist. Surprisingly, the observed size of the berberine is of the same order, much larger than size

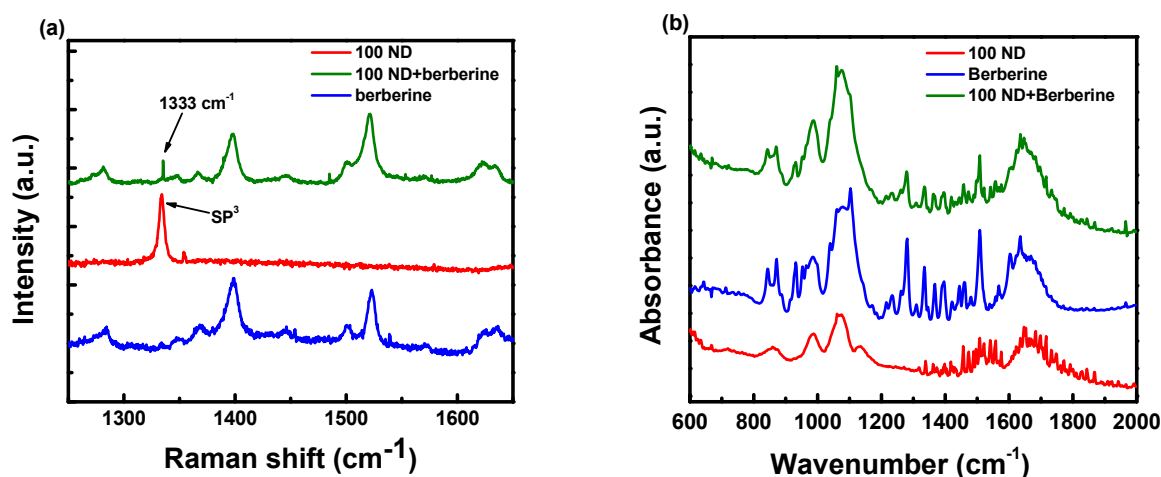


Fig. 2 Spectra of ND, berberine and ND-berberine complex: (a) Raman; (b) FTIR.

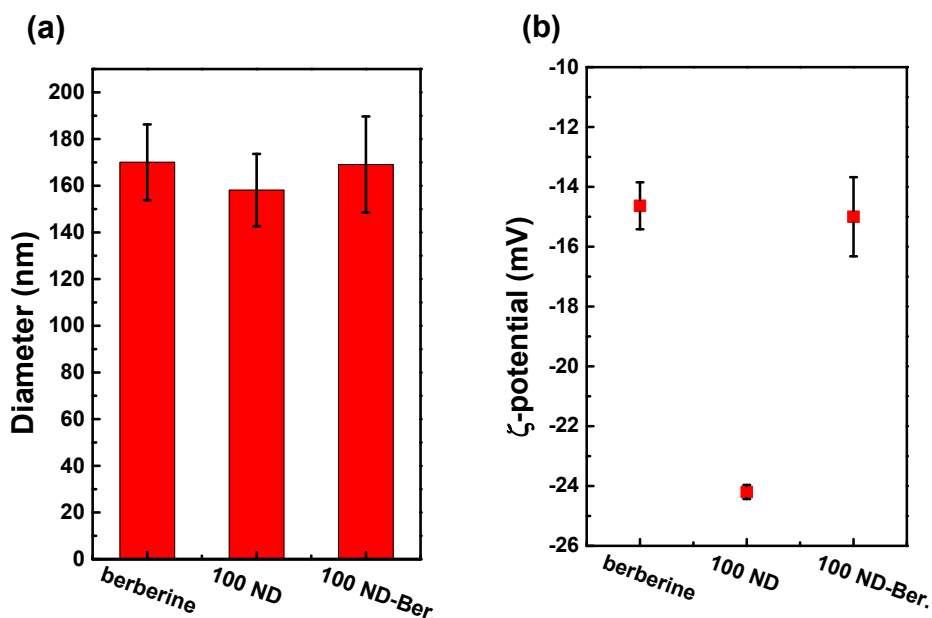


Fig. 3 The particles size (a) and zeta-potential (b) measured at pH~7.45.

of the berberine molecules. Berberine is known not very well soluble in water, so in this experiment we could have observed residual nanocrystals of the berberine, not molecules. The much larger size of the berberine can be a concern, but we follow our protocol to prepare ND complex with bio-molecules and didn't change it in this work except heating for comparison. We first prepare solution of the adsorbed molecules and then add ND. In this case we can estimate how much the bio-molecules are adsorbed. High speed centrifuging does reduce the berberine size, but we will lose the information on the concentration. However, when ND-berberine is formed, the complex size is within expectation, meaning the non-dissolved berberine did not participate in the reaction. The changes of ζ -potential (Fig. 3b) from about -24 mV for 100 nm cND to -15 ± 2 mV for ND-berberine complex almost the same as the measured value for pure berberine, indicates

the berberine modifies the ND surface to change the surface charges distribution. Judging from the size, ND-berberine is formed with ND core and adsorbed berberine molecules (not crystal or cluster); while looking at the charge, ND-berberine forms overall charge similar that of clustering berberine also indicating we do have a layer of berberine on the ND. The complex charge of ND-berberine is determined by layers of berberine.

From Fig. 1, the intensity changes in the absorption spectra after the berberine adsorption on ND, and the measured size of the ND-berberine complex (in Fig. 3, close to ND) as well as the observed ND peak in Raman spectrum (Fig. 2b) allow confirming the formed ND-berberine complexes are ND particles with adsorbed berberine molecules on the surfaces. Both ND and berberine have negative ζ -potential; this can explain the

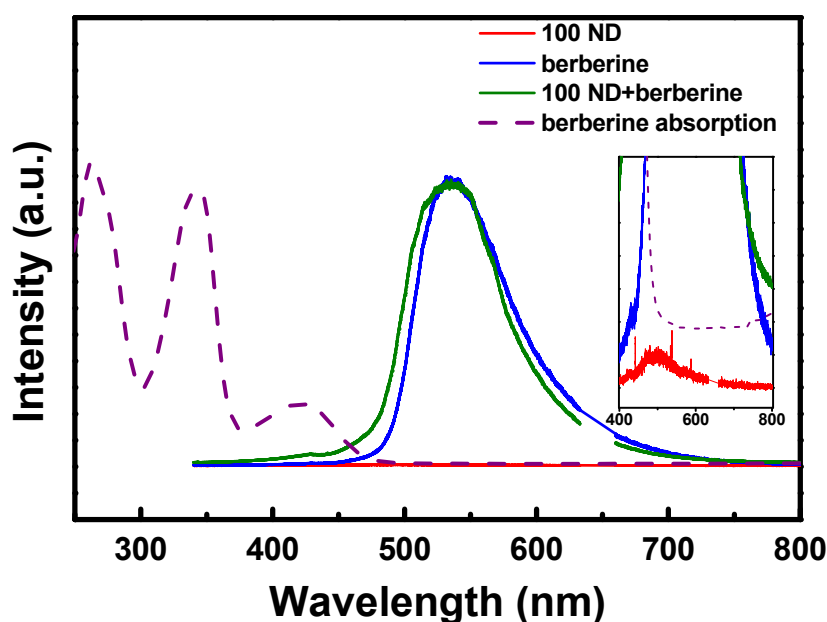


Fig. 4 Photoluminescence spectra of ND, berberine and ND-berberine complex (shown in solid lines) and spectrum of UV-Visible absorption of berberine (shown in dash line). Inset: extended spectrum of ND.

low stability of the complex in washing, observed via absorption spectra (Fig. 1).

Nanodiamond fluorescence is predominantly based on defect color centers in diamond structure. It has a number of advantages, such as stability, can be excited in wide range of excitations frequencies, etc. It is promising for imaging in bio-applications, including tracing of delivery of non-fluorescent substances (such as many drugs) [5]. In the present work we use highly fluorescent berberine, therefore we can simultaneously observe both the ND and berberine in the cellular model.

Photochemical properties of berberine have recently been investigated. The fluorescence of berberine can serve as fluorescent marker. In addition, both absorption and emission of berberine are sensitive to environment and can be used for sensing [34, 37-42]. Fluorescence spectra of berberine, ND, ND-berberine complex are shown in Fig. 4, together with absorption spectrum of berberine solution in PBS. The spectra were excited with 325 nm wavelength excitation laser where the energy is close to the berberine absorption maximum. The measured peak is intense, structureless and slightly asymmetric with a maximum near 535 nm. Blue shift about 7 nm and slight change of the peak shape can be observed when berberine is adsorbed on ND surface. We can also observe (qualitatively) enhancement of adsorbed berberine fluorescence but can't estimate it quantitatively using our dried samples.

The intensity increasing and the shift of the fluorescence maximum of berberine when berberine interacts with environment have been described and analyzed previously, using non-polar solvents [34], DNA [37, 42] and many other bio-organic molecules and systems [e.g. 41]. The mechanisms are discussed, particularly, it was supposed that the interaction of

berberine with these systems reduces the interaction with water (polar solvent) and hinders relaxation mechanism of the excited berberine [37]. Oppositely, effect of berberine derivative on quantum dot (QD) CdTe nanoparticle luminescence properties has been studied and it has been suggested that berberine adsorbing on the QD surface is responsible for disrupting electron-hole recombination process and resulting in the repression of luminescence [39].

The fluorescence image of 100 ND-berberine complexes is shown in Fig. 5. The fluorescence of 100 nm ND is excited with 488 nm wavelength laser and collected in the 500-530 nm wavelength range (Fig. 5 (a)). The fluorescence of ND in this range corresponds predominantly to emission from diamond H3 defect centers with emission peak near 505 nm [43]. Berberine fluorescence was excited with 405 nm wavelength laser, corresponding to absorption band of the berberine. The signal was collected in the 530-620 nm spectral range; this region includes maximum and long-wavelength part of berberine fluorescence (Fig. 5 (b)). Fig 5c displays the merged (a) and (b) images, revealing the large (dried) aggregate of ND with adsorbed berberine. In addition, adsorption of macromolecules (such as berberine in this case) can slightly change the photoluminescence of the observed spectrum (Fig. 4), but does not affect the imaging [44, 45]. So, wide application of berberine in biophotonics can be expected due to its bright fluorescence, even in polar solvent like PBS, which can be used to observe the delivery into cell and localization of the drug inside in comparison with ND-drug complex.

Confocal images of A549 cell at their interaction with berberine and ND-berberine complex are presented in Fig. 6. In Fig. 6 (I), fluorescence images of A549 after 2 h incubation in berberine solution are presented.

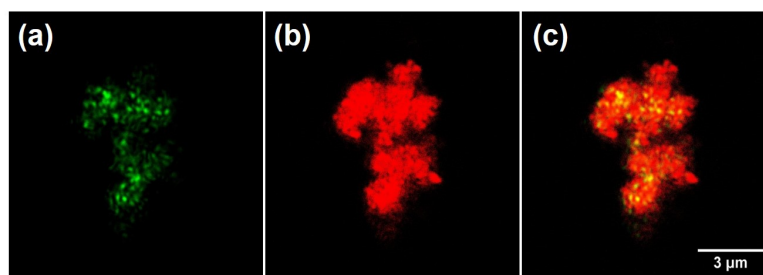


Fig. 5 Fluorescence image of aggregated 100 ND-Berberine complex: (a) 100 nm ND fluorescence excited with 488 nm wavelength laser, signal collected in the 500-530 nm range, shown in green color; (b) berberine fluorescence excited with 405 nm wavelength laser, signal collected in the 530-620 nm spectral range, shown in red color; (c) merged image of (a) and (b).

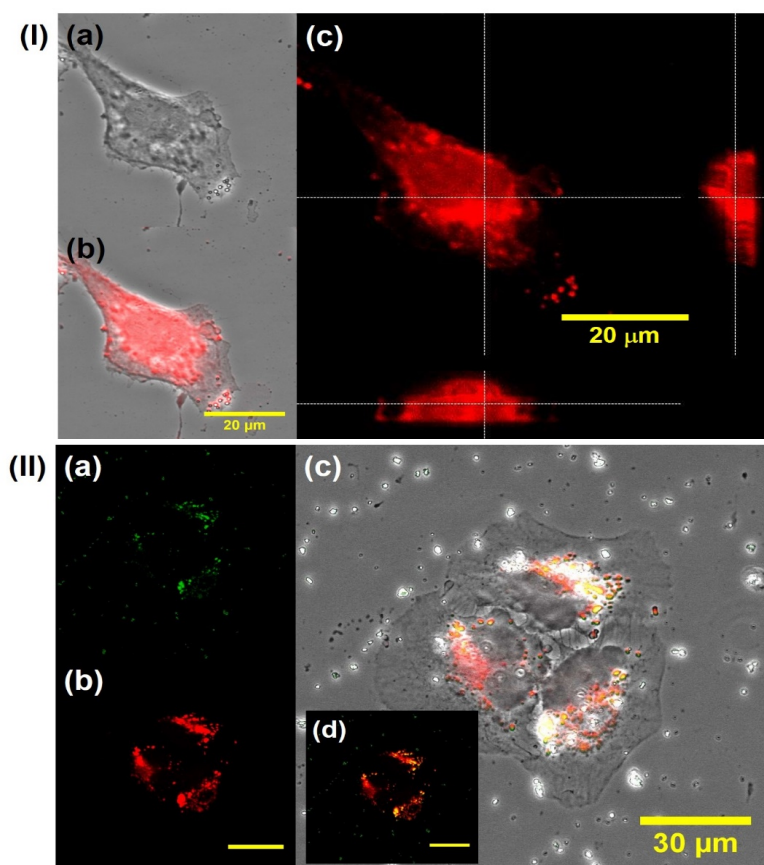


Fig. 6 Confocal imaging of A549 cell interaction with berberine and ND-berberine complex. (I) A549 with berberine: (I-a) Bright field optical image, (I-b) merged image of fluorescence and bright field optical images. (I-c) The x-z and y-z projections of the z-scan of A549 with berberine. The z-scan is measured with a 250 nm step. (II) A549 with ND-berberine complex. (II-a) Image of ND, fluorescence is excited with 488 nm wavelength laser and signal is collected in the 500-530 range; (II-b) image of berberine, fluorescence is excited with 405 nm wavelength laser and signal collected in the 530-620 nm spectral range. (II-c) Merged (II-a), (II-b) and bright field optical images. (II-d) is merged (II-a) and (II-b) fluorescence images.

Comparison of bright field optical image (I-a), fluorescence optical image (I-b) and analysis of the z-scanning (I-c) allow observation the berberine's penetration inside and distribution in the cell. In Fig. 6(II) the images of A549 interacting with ND-berberine are shown. We also have compared these images with the cell images after incubation with 100 nm ND (not shown) which demonstrate observed previously distribution of ND in the cell cytoplasm [13, 14]. ND-

berberine complex also is localized in the cytoplasm; the signals from ND (Fig. 6 (II-a)) and berberine (Fig. 6 (II-b)) are observed colocalized and overlapped (Fig. 6 (II-c)), so berberine is still not released from ND. While the observed distribution of ND and ND-berberine complex in the cell cytoplasm is similar after 2 h incubation (Fig. 6 (II)), berberine distribution in the cell is more homogeneous and it can gradually penetrate into nuclei (Fig. 6 (I)). Note that at longer incubation time

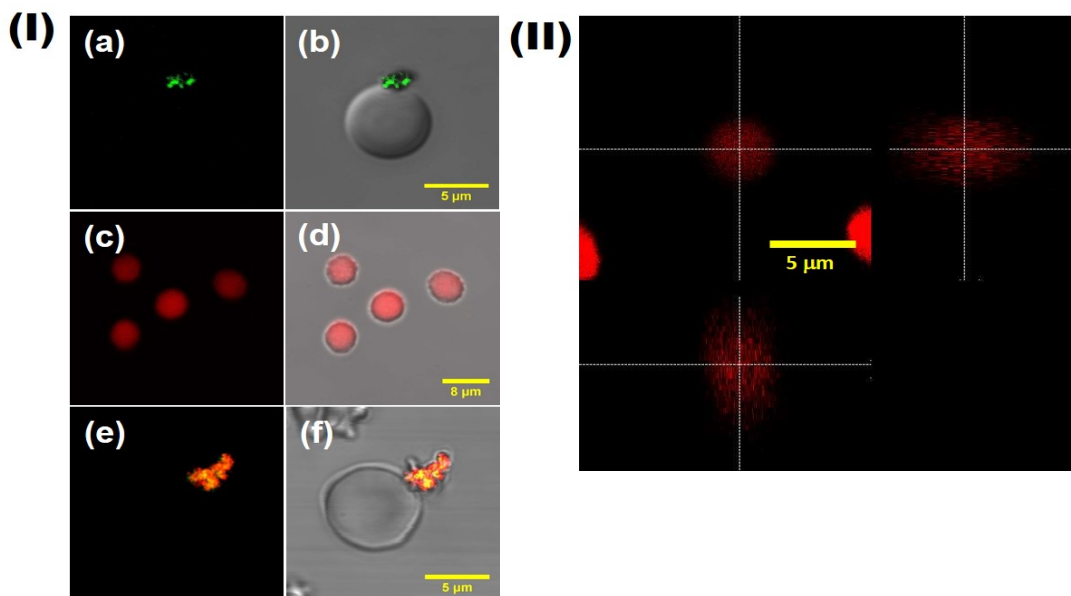


Fig. 7 (I) Fluorescence imaging of RBC interaction with 100 ND (a-b), berberine (c-d) and ND-berberine complex (e-f). (II) The x-z and y-z projections of the z-scan of RBC with berberine. The z-scan is measured with a 400 nm step. The ND fluorescence is excited with 488 nm wavelength laser and signal is collected in the 500-530 nm range (shown in green color). The berberine fluorescence is excited with 405 nm wavelength laser and signal is collected in the 530-620 nm spectral range (shown in red).

(from 48 h) berberine is colocalized with ND in less degree and can be observed in the nuclei area (not shown). But ND never penetrates into nuclei up to incubation of 48 h and more.

The works using nanoparticles (NP) containing or attaching berberine, first of all, are intended to overcome its low solubility, protect berberine from destruction by gastric acids, provide controllable release [46]. For that purpose chitosan porous particle [47], polymer [48] and lipid encapsulation [49] are usually used. Moreover, using nanoparticles, particularly ND, for targeted drug delivery expects increasing efficiency of the treatment [2, 50] utilizing various mechanisms. Among the discussed mechanisms [18-20], different pathways of the internalization by cells and controlled release are considered. Thus, Iron oxide [50, 51], gold [51], silica [52], graphene [53, 54] NP have been suggested as berberine carrier. The enhanced anticancer efficiency of SiO₂-berberine complex has been observed [52]. It has been hypothesized that an increase in local concentration due to the confinement of a ligand on the nanosurface ("dendritic" effect) might have led to the observed effect. Unique properties of carbon-based NP, graphene and graphene oxide, are utilized to obtain new berberine carrier demonstrated as a system for anticancer drug delivery and fluorescent probe for multi-excitation [53]; graphene oxide electric and electrochemical properties were utilized for development of system allowing electric-sensitive drug release and enhanced oxidation of berberine as a model of metabolic regulation of drugs according to the different electrophysiological environment in tumor therapy [54]. These works show that potential of

nanoparticles in the berberine applications and involving berberine investigations is high.

For further in-vivo applications, delivery of ND-drug complex to the targets via the blood flow will be a natural step. Therefore, the interaction of ND with the blood system is an important subject to study. In this work, in-vitro analysis of ND-berberine interaction with RBC was also performed. The microscopic fluorescence images of RBC after incubation for 30 min with ND, berberine and ND-berberine are presented in Fig. 7. In the Fig. 7 (I-a, b) ND fluorescence (shown in green) indicates the NDs are only attaching to the RBC cell membrane. The RBC incubated with berberine solution (in PBS solvent) is shown in Fig. 7 (I-c, d). The fluorescence is observed (shown in red) as a result of the berberine penetration into RBC. Fluorescence of berberine inside the RBC can be seen in the x-z and y-z projections of the z-scan (Fig. 7 (II)). For the ND-berberine incubation with RBC, its interaction with RBC is similar as for the ND. The images in Fig. 7 (I-e, f) show ND-berberine attached on the RBC membrane. Thus, we confirm the controllable localization of berberine in complex with ND on certain cellular structures.

We also have compared the effect of ND and ND-berberine complex on RBC function using *in situ* Raman spectroscopic measurements; method has been presented previously [16, 17]. RBC Raman spectra are determined by Hb inside and are very sensitive to the oxygenation and spin state dynamics of the heme (the active center of Hb) due to a selective enhancement of the Raman active vibrations in different oxygenation states [55]. To check in a first approximation whether

berberine adsorbed on ND's surface affects RBCs' function, Raman spectra of a single RBC incubated for 30 min with ND, berberine and ND-berberine have been measured at oxygenated and deoxygenated states (Fig. 8). The spectra of RBC in oxygenated state are shown in Fig. 8a, the RBC were oxygenated by air. Then, the same cells were deoxygenated by N_2 purging the sample; the spectra are shown in the Fig. 8 (b). Finally, the cells were oxygenated again by air (Fig. 8c). In the process of deoxygenation and oxygenation the spectra exhibit significant changes. The major differences in the spectra are attributed to changes in the spin state of heme Fe and to the following conformational changes in Hb molecules [55]. The oxygenation degree of the

samples can be monitored using the oxidation state marker bands, for example, ν_4 in the range 1355 to 1380 cm^{-1} , sensitive to the spin and coordination state of the heme, its changes are marked in the Fig. 8. Since RBC is relatively larger than berberine or ND-berberine, when it attaches to the RBC membrane, it did not affect the RBC oxygenation/de-oxygenation function. We didn't observe effect of berberine and ND-berberine complex on the RBC spectra both in oxygenated and deoxygenated state, as well as after repeated oxygenation. Thus, we can conclude that neither berberine, nor ND-berberine complex observably affect RBC state and ability to transfer oxygen.

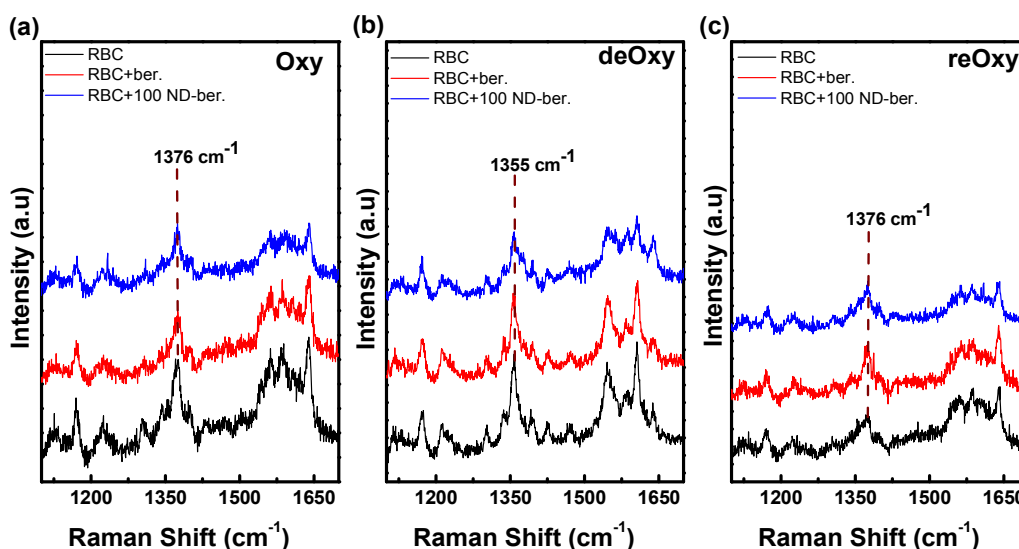


Fig. 8 Effect of berberine and ND-berberine complexes on Human Red Blood Cells: The Raman spectra of the RBC in oxygenated (a) deoxygenated (b) re-oxygenated states.

4 Conclusion

In this study the interaction of ND-berberine complex with human A549 cancer cell and human RBC are studied. The berberine was selected for its fluorescent properties, to observe simultaneously the nanodiamond and the berberine. The distribution of berberine, ND and ND-berberine relatively in cellular structures has been observed using independently ND and berberine fluorescence signals for detection. It is shown that ND-berberine complex penetration into cell and the distribution of the berberine in the cell organelles/structures is determined by ND. It allows controlling the berberine interaction with cell by attaching it on ND. We also show the interaction of ND,

berberine, and ND-berberine doesn't affect the oxygenation properties of the human red blood cells. When connected to ND, the berberine distribution is determined by the ND, and attached to the cell membrane of RBC. This study also demonstrates the possibility of controlling the drug internalization in target cell which may increase the efficiency of drug treatment.

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Characterization of porous media based on the polarimetric matrix models

Sergey Savenkov¹, Alexander Priezzhev², Yevgen Oberemok¹, Ivan Kolomiets¹, Pertti Silfsten³, Tuomas Ervasti⁴, Jarkko Ketolainen⁴, and Kai-Erik Peiponen³

¹ Faculty of Radio Physics, Electronics and Computer Systems, Taras Shevchenko National University of Kyiv, 64 Vladimirskaya str., Kiev 01033, Ukraine

² Department of Physics and International Laser Center, Lomonosov Moscow State University, Vorobyovy Gory, Moscow 119992, Russia

³ Department of Physics and Mathematics, University of Eastern Finland, PO Box 111, FI-80101 Joensuu, Finland

⁴ School of Pharmacy, Promis Centre, University of Eastern Finland, PO Box 1627, FI-70211 Kuopio, Finland

* e-mail: sns@univ.kiev.ua

Abstract. In this paper, we measured the Mueller matrices of the samples with different porosity in the visible range (the wavelength of the input radiation 632.8 nm) for the observation angles from 5° to 85° . The samples under study were the tablets made of microcrystalline cellulose with the known values of porosity. To characterize the depolarizing properties of the studied samples we used the matrix models of depolarizing medium, in which the Mueller matrices describing depolarization have different structure and include different numbers of parameters. It is shown that Mueller polarimetry ensures at least 5% accuracy of porosity identification for this class of objects at the wavelength 632.8 nm. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Mueller matrix; porosity; depolarization; linear birefringence; circular birefringence.

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1 Introduction

The results recently obtained in polarimetry clearly demonstrate the availability of polarimetric methods for studying the properties of biological objects [1-3]. It is known that biological objects are characterized by complex “polarization behavior” [2]. In particular, the objects of this class can demonstrate both linear and circular birefringence and depolarization. The interaction of polarized electromagnetic radiation with biological objects is characterized by high depolarization, and, more importantly, depends on the input polarization state.

The dependence of depolarization of the output radiation on the input polarization state is referred to as anisotropic depolarization. The most common method to characterize the depolarizing properties of objects is to use the so-called single value depolarization metrics [4]. All existing depolarization metrics can be divided into two classes. The first class is the metrics including only the elements of the Mueller matrix of the studied object, e.g., the depolarization metric [5], the Q -metric [6], the Cloude [7] and Lorentz [8] entropy. The second class is the metrics involving the scanning of all possible states of input polarizations, see, e.g., Ref. [9].

It should be borne in mind that the single value depolarization metrics carry some averaged information on the depolarization. This means that in case of anisotropic depolarization the information capabilities of single value depolarization metrics appear to be limited. Moreover, in the literature the cases are described, when certain metrics generally do not adequately characterize the depolarizing properties of the studied object [8].

Therefore, to characterize the polarization properties of biological objects and, in particular, their depolarization capabilities, the matrix models of depolarizing medium show considerable promise.

In this case, the Mueller matrix of the object is represented as a matrix product of, using the terminology Jones [4], elementary types of anisotropy, i.e., the linear and circular birefringence and dichroism, and the matrices describing depolarization. In Refs. [10-12] the authors demonstrated the possibility of a matrix model based on the so-called generalized polar decomposition theorem [13] to obtain information on linear and circular birefringence in high depolarization of the output radiation.

In this paper, we study the sensitivity of the Mueller method and various depolarization matrices to the porosity of samples, which are tablets made of a

common pharmacological filler, the microcrystalline cellulose [14]. The paper is organized as follows. Section 2 outlines the existing Mueller matrix models of depolarizing objects. In Section 3 the detailed information about the porous samples under consideration is presented. Section 4 presents the results of measurements, comparative analysis and discussion.

2 Matrix models of depolarizing objects

The propagation of electromagnetic radiation in an inhomogeneous medium, alongside with the change in the polarization state, is also accompanied by the "removal" of a portion of the input radiation energy, which is spatially redistributed over the different directions of propagation, i.e., the radiation is scattered [15-17]. The scattering is usually accompanied by the depolarization of the output radiation [15, 18]. The depolarizing capability is a property of the studied object, which manifests itself as a decrease of the polarization degree of the input completely polarized radiation after its interaction with the object.

There are two types of matrix models of depolarizing objects, the additive and the multiplicative ones. The additive matrix models are associated with the discrete approximation of the medium, i.e., the studied medium is represented as a set of scatterers. These models are based on the weighted summation of deterministic Mueller matrices describing the interaction of radiation with individual scatterers [19, 7]. In this paper, this type of model is not considered.

The multiplicative models represent the studied medium (deterministic and/or depolarizing) as a structure consisting of a number of layers, successive with respect to the propagation direction of the input radiation. Each layer is described by the Mueller matrix of only one type of anisotropy and/or depolarization [20].

Apparently, the first multiplicative matrix model of depolarizing medium was the model proposed in Ref [21]. In this model the Mueller matrix of depolarizing medium is presented as a product of deterministic Muller matrices, describing linear birefringence, linear dichroism, etc., and the depolarization matrix of the form

$$\begin{pmatrix} 1 & A_1 & A_2 & A_3 \\ 0 & P_1 & 0 & 0 \\ 0 & 0 & P_2 & 0 \\ 0 & 0 & 0 & P_3 \end{pmatrix}. \quad (1)$$

The diagonal elements of the matrix (1) represent the degree of the following input polarizations: horizontal and vertical, linear with the azimuths $\pm 45^\circ$, and the left and right circular ones. The quantities A_1 , A_2 , and A_3 in the first row of the matrix (1) are quantitative estimates of depolarization asymmetry for the above three pairs of orthogonal input polarizations.

The model most widely used to date is the multiplicative model of depolarizing medium proposed in Ref. [13]. In this model $\mathbf{M} = \mathbf{M}_\Delta \mathbf{M}_R \mathbf{M}_D$, where $\mathbf{M}_R$ is the matrix of a retarder, $\mathbf{M}_D$ is the matrix of a diattenuator, and the matrix:

$$\mathbf{M}_\Delta = \begin{pmatrix} 1 & \bar{\mathbf{O}}^T \\ \bar{\mathbf{P}}_\Delta & \mathbf{m}_\Delta \end{pmatrix}, \quad \mathbf{m}_\Delta^T = \mathbf{m}_\Delta, \quad (2)$$

describes the depolarizing properties of the studied object. This model is referred to as the generalized polar decomposition. In Eq.(2) the vector $\bar{\mathbf{P}}_\Delta$ describes the polarization state "generated" by the Mueller matrix (2), when the input radiation is unpolarized.

In Ref. [23] the model of depolarizing medium, described by the symmetrical expansion of its Mueller matrix, is proposed:

$$\mathbf{M} = \mathbf{M}_{D2} \mathbf{M}_{R2} \mathbf{M}^{DA} \mathbf{M}_{R1}^T \mathbf{M}_{D1}, \quad (3)$$

where the symbol " T " denotes the transposition of the matrix $\mathbf{M}_{R1}$; $\mathbf{M}_{D1}$, $\mathbf{M}_{D2}$ and $\mathbf{M}_{R1}$, $\mathbf{M}_{R2}$ are the matrices of two elliptical polarizers and phase plates, respectively; the matrix $\mathbf{M}^{DA}$ is a depolarization matrix having the form

$$\mathbf{M}^{DA} = \begin{pmatrix} m_{11} & 0 & 0 & 0 \\ 0 & m_{22} & 0 & 0 \\ 0 & 0 & m_{33} & 0 \\ 0 & 0 & 0 & m_{44} \end{pmatrix}. \quad (4)$$

One can see that the depolarization matrices (1), (2), and (4) have different structures and contain a different number of parameters. The analysis aimed at the interpretation of the physical properties of depolarizing objects based on the above matrix models [13, 21, 22] is beyond the scope of this paper. Below we use the depolarization matrices (1), (2), and (4) to assess their sensitivity to the porosity degree of the considered samples.

3 Objects of study and experimental technique

In this paper, we investigate the porous samples made in the form of tablets [14]. The tablets (the diameter 13 mm, the thickness 2 mm, and the porosity 40.1%; 35.1%; 30.7%; 26.7%, and 23.2%) were made using the press PUUMAN PCS-1 from microcrystalline cellulose (Avicell PH-200 FMC Biopolymer), a filler commonly used in pharmaceutical industry to produce tablets.

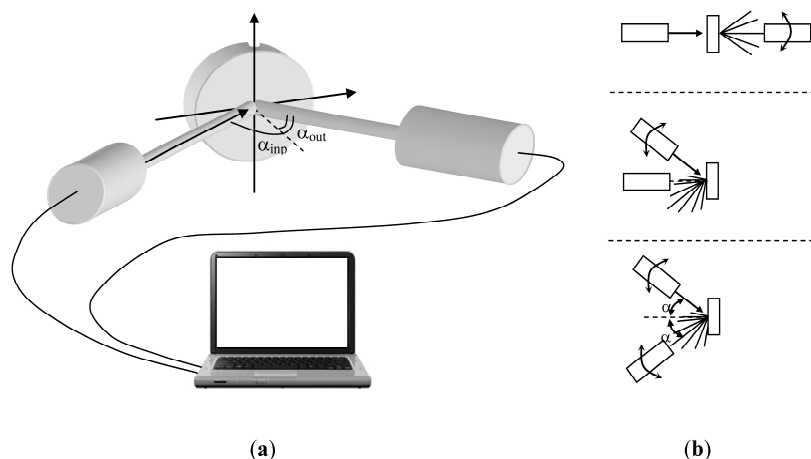


Fig. 1 (a) Schematic diagram of the Mueller polarimeter; (b) geometry of experiment.

The compression was implemented using the cylindrical punches. The variation of the sample porosity was achieved by changing the cellulose powder mass in the range from 397 mg to 512 mg with simultaneous change of the upper punch contour, providing the stability of the sample thickness. The compression speed was 100 mm/s. After one day of compression, the dimensions and weight of the tablets were measured, making it possible to calculate the density of each sample. The absolute density of samples was determined using the pycnometer Quantachrome MVP-1.

The schematic diagram of the Mueller polarimeter is shown in Fig. 1a.

The polarized electromagnetic radiation was generated by the He-Ne laser ($\lambda = 632.8 \text{ nm}$). The input channel was composed of a fixed prism polarizer and two phase plates, implemented as controlled liquid crystal cells with different azimuths of orientation and values of the phase shift.

The receiving channel consisted of the continuously rotating crystal phase plate, the fixed prism polarizer orthogonal to the one in the input channel, and a photodetector. Thus, the receiving channel was actually a full-Stokes polarimeter [23].

During the measurements, various options of the experimental geometry were tested (see Fig. 1b). The geometry with similar input and observation angles (the lower part of Fig. 1b) appeared to be the most informative. For the geometry shown in the top part of Fig. 1b, the measurement at the wavelength $\lambda = 632.8 \text{ nm}$ and the sample thicknesses of 2 mm are also possible. However, in this case the output radiation is completely depolarized for all the states of the input polarization.

For all five samples we have measured the complete Mueller matrices in the range of observation and input angles from 5° (this value was determined by the design constraints of the polarimeter receiving channel) to 85°

(for the observation angles $85^\circ - 90^\circ$ the intensity of scattered radiation is vanishingly small).

Before the measurement in each sample, the calibration measurements of the Mueller matrix were carried out in test objects, i.e., the empty space, the prism polarizer, etc. The Mueller matrices for each of the observation angles, analyzed in the next section, were obtained as a result of averaging over five hundred measurements. The measurement error of the matrix elements did not exceed 1%.

4 Results and Discussion.

Based on the measured Mueller matrices, the depolarization matrices (1), (2) and (4) were calculated for all observation angles. Figures 2 a, b, and c present the non-zero elements versus the observation angle for the matrices (1), (2) and (4), respectively.

As seen from Fig. 2, at small observation angles, $5^\circ - 25^\circ$, the situation is close to the isotropic depolarization, which is to the isotropic factor described by the following Mueller matrix

$$\mathbf{M} = \begin{pmatrix} 1 & 0 & 0 & 0 \\ 0 & 0.2 & 0 & 0 \\ 0 & 0 & 0.2 & 0 \\ 0 & 0 & 0 & 0.2 \end{pmatrix}. \quad (5)$$

The possible explanation is that the multiple volume scattering dominates in this case and the degree of polarization of the output radiation is only slightly dependent on the input polarization state.

At large observation angles, $70^\circ - 85^\circ$, all depolarization matrices (1), (2), and (4) degenerate into the unit matrix. The reason is that in this case the single scattering from the surface of the tablets is dominant, and the depolarization of the scattered radiation is minimal or absent.

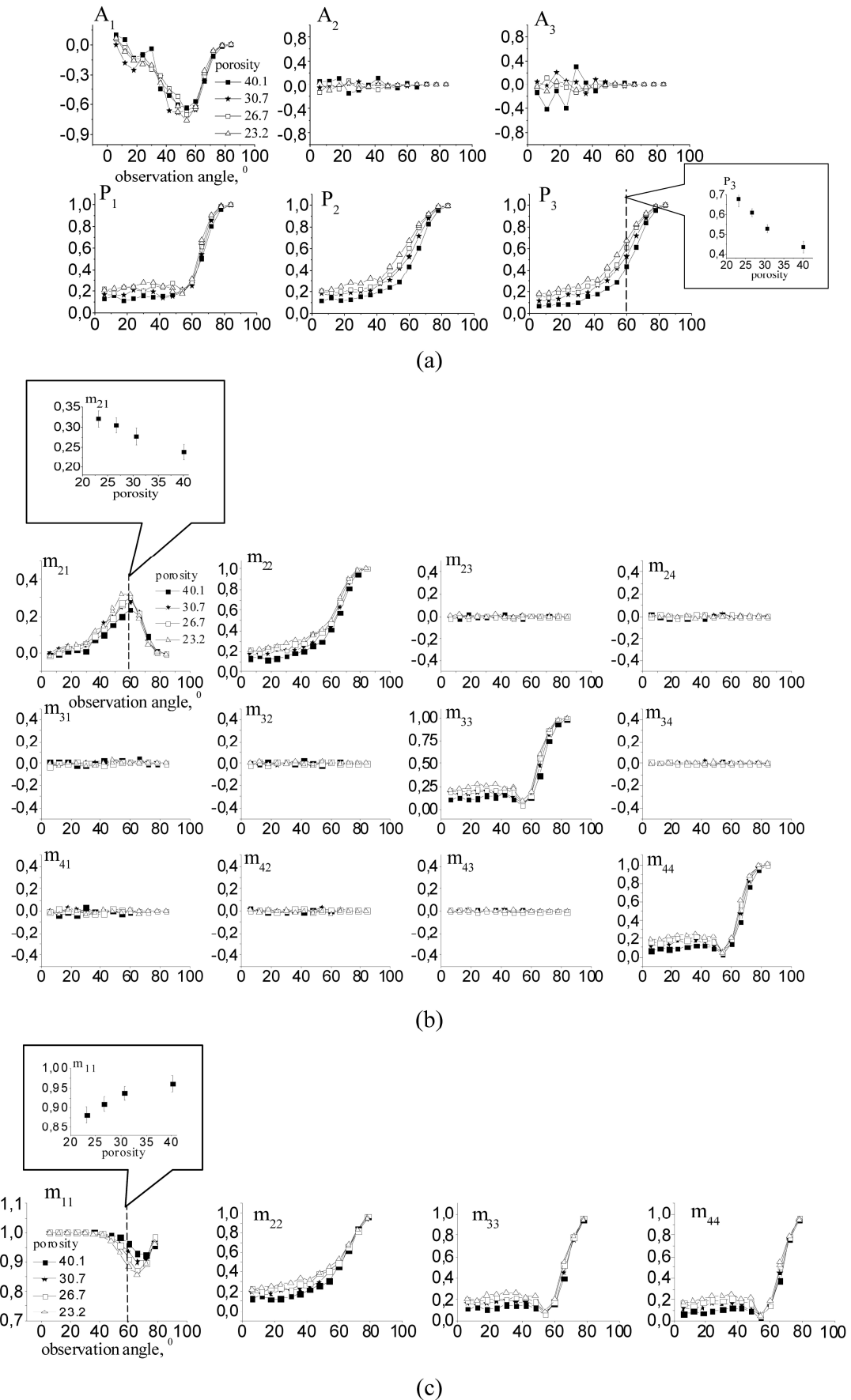


Fig. 2 Dependence of nonzero elements of the matrices (1), (2), and (4) on the observation angle.

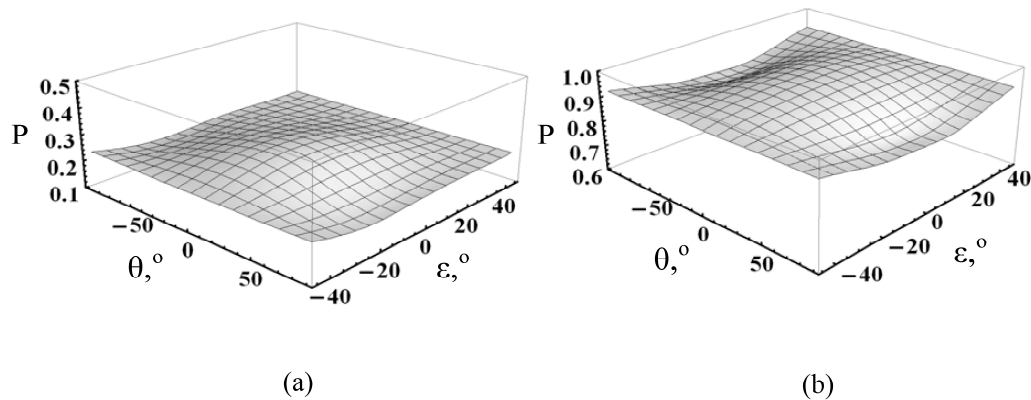


Fig. 3 Dependence of the output degree of polarization on the azimuth θ and the ellipticity ε of the input polarizations for the sample with the porosity of 23.2% and the observation angles 18° (a) and 72° (b).

Note that for the depolarization matrices (1) and (2), having no nonzero off-diagonal elements, only the elements m_{12} in the matrix (1) and m_{21} in the matrix (2) vary depending on the observation angle. For the diagonal elements m_{22} , m_{33} , and m_{44} the dependence on the observation angle is quite similar and determined by the interrelation between the volume and the surface scattering for a given observation angle.

In our opinion, this results from the fact that the depolarization, by which the samples under consideration are characterized, is weakly dependent on the input polarization. In other words, in this case, the depolarization is weakly anisotropic. This fact is illustrated by the dependences of the output degree of polarization on the azimuth and ellipticity of the input polarizations, presented in Fig. 3.

For the depolarization matrix (2), if the input electromagnetic radiation is unpolarized, then the output radiation is partially polarized linearly and horizontally. The maximal value of the polarization degree varies

from 25 % to 35 %, depending on the porosity, and is achieved within the range of observation angles from 50° to 60° .

In each of the cases, illustrated by Figs. 2 and 3, there are matrix elements and ranges of observation angles that allow reliable identification of samples having different porosity. However, the sensitivity to the sample porosity is rather different in the considered models (see the corresponding insets in Figs. 2 and 3).

5 Conclusion.

The obtained results demonstrate that the Mueller polarimetry ensures the identification of the porosity for a given class of objects at the wavelength $\lambda = 632.8$ nm of the input radiation with the accuracy better than 5%. The next step would be to conduct similar studies for other wavelengths of the input electromagnetic radiation.

Multimodal texture analysis of OCT images as a diagnostic application for skin tumors

Dmitry S. Raupov^{1*}, Oleg O. Myakinin¹, Ivan A. Bratchenko¹, Valery P. Zakharov¹, and Alexander G. Khramov²

¹ Dept. of Laser and Biotechnical Systems, Samara National Research University, Russian Federation

² Technical Cybernetics Dept., Samara National Research University, Russian Federation

* e-mail: radim94@mail.ru

Abstract. Optical coherence tomography (OCT) is an effective tool for determination of pathological topology that reflects structural and textural metamorphoses of tissue. In this paper, we propose a report about our examining of the validity of OCT in identifying changes using a skin cancer texture analysis compiled from Haralick texture features, fractal dimension, complex directional field features and Markov random field method from different tissues. The experimental data set contains 530 OCT images with normal skin and tumors as Basal Cell Carcinoma (BCC), Malignant Melanoma (MM) and Nevus. Speckle reduction is an essential pre-processing part for OCT image analyze. In this work, we used an interval type-II fuzzy anisotropic diffusion algorithm for speckle noise reduction in OCT images (B- and/or C-scans). The Haralick texture features as contrast, correlation, energy, and homogeneity have been calculated in various directions. A box-counting method and other methodologies have been performed to evaluate fractal dimension of skin probes. The complex directional field calculated by the local gradient methodology provides important data for linear dividing of species. We also estimated autocorrelation function using Markov random fields. Additionally, the boosting has been used for the quality enhancing of the diagnosis method. And, finally, artificial neural network (ANN) has been utilized for comparing received rates. Our results demonstrate that these texture features may present helpful information to discriminate a tumor from healthy tissue. We obtained sensitivity about 92% and specificity about 95% for a task of discrimination between MM and healthy skin. Finally, a universal four classes classificatory has been built with average accuracy 75%. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: optical coherence tomography; texture analysis; Haralick features; fractal dimension; complex directional field; diffuse filter; Markov random field.

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1 Introduction

Optical Coherence Tomography (OCT) is a great tool for contactless nondestructive study of optically inhomogeneous mediums [1]. Recently, effective application of OCT has been confirmed in various directions of clinical practice such as gastroenterology, urology, dermatology, gynecology, ophthalmology, otolaryngology, stomatology and others [2, 3]. OCT has a position as a non-invasive method of visualization of inner structure of optically heterogeneous objects based on a principle of low-coherence interferometry using near infrared range (0.75–1.3 μm) as light source. OCT visualizes an inner microstructure of skin down to 2 mm with space resolution 10-15 μm without human tissue invasion [4]. Optical coherence tomography is usually employed for a measurement of structural changes of tissue. The possibility of OCT in detecting changes using Haralick's texture features, fractal dimension and complex directional field extracted from different tissues has been investigated in this paper.

The main goal of this work is the development of a multimodal method for the texture analysis of OCT images. As is well known, one of the most dangerous neoplasms, the malignant melanoma, has a large absorption coefficient, which means the OCT image has a very low level of signal-to-noise ratio (SNR) in the region of the neoplasm. Thus, using only one textural

technique is a priority not enough. However, combining texture modalities can guarantee accuracy improvement [5].

Any coherent method implies speckle noise impact for the imaging. In general case, the noise can be removed by digital filter from final OCT volume after a mandatory PC processing [6]. On a 2D slice of tissue volume in vertical (B-scan) or horizontal (C-scan) planes, it is available to see dynamics of anisotropic growth for malicious area and following deformation for encircling healthy one. It is just an image in terms of Digital Image Processing and the denoising by way of a general image processing filter [7, 8] must be applied. We used an interval type-II fuzzy anisotropic diffusion algorithm [9, 10] for speckle noise reduction in OCT images for this research as more reliable for processing on OCT images.

Haralick texture features have been evaluated in different directions as an initial and basic solution for the multimodal approach. These quickly computable features contain useful information for categorizing, classifying diagnostic images as B-scans. However, in biology and medicine, the shapes of structures such as molecules, cells, tissues and organs also play an important role in the diagnosis of diseased tissue [11, 12]. Fractal dimension could also distinguish the structural changes of tissue. Quantitative evaluation of

the fractal dimension could be an efficient concept to differ tumor tissue from normal healthy tissue [13, 14]. So the structural changes in fractal dimension may give further information observing cellular layers and injury in skin pathology. Besides, the using of the OCT techniques contributes to the application of fractal analysis in the diagnostics of skin cancer. The suspicious skin tissue could be revealed by accomplishment of fractal analysis for certain biological structures in OCT images. Thus, fractal analysis with the OCT imaging techniques could give an efficient diagnostic methodology to classify tumors as BCC and MM [15]. Also, it can be used for differentiation between nevus and healthy skin probes. Usually, we can simplify numerical dependencies in tissue topology elementary volumes (planes, specifically, if we are talking about images) by suggestion, that volume depends from only her neighbor ones. It means, that a computing, for instance, Markov random fields' [16] features could be helpful to provide us useful information about nature of tumor.

We included a method of the complex directional field [17] to this features' list to improve the characteristics' quality of tumor recognition. Malignant tissue growth anisotropically, which means it may be interpreted by the complex directional field calculated from a C-scan. General features like variance and correlation could be used for dividing scans with tumor and healthy probes. A basic idea of a directional field application is an attempt to analyze the directions of anisotropic growth of malicious skin and possibility of tissue further development. High relevant results on quasiregular structures such as interferograms, fingerprints, crystallograms and many others show us its universality [17]. The complex form provides more evaluation precision and a relationship between a directional field and a weight function. The C-scan analyzing gives us an ability to appreciate other planes of spreading pathology on the cellular layers.

Unfortunately, we also should take into account additional a priori information about preliminary diagnosis when we use textural features for common dividing between two classes (MM or BCC, MM or Nevus etc.). Thus, it could be a big challenge for general practitioner. So, it is very important to have a universal classifier for accurate definition between many tumor classes. A priori information is also required for this case, but preliminary diagnosis could be much less accurate. The boosting [18] is used in this work with aim to generalize all textural features, enhance diagnosis quality and receive classifier for discriminating between 4 classes (MM, BCC, Nevus or healthy skin). Additionally, we tested ANN as an alternative method of machine learning to create a linear combination of described textural features for increasing sensitivity and specificity.

2 Materials and Methods

2.1 OCT-setup scheme and tissue samples

The OCT system (Fig. 1) comprises of a broadband superluminescent laser diode (840 ± 45 nm wavelength range, 14mW output power) at the source end, Michelson interferometer with 50/50 split ratio to the sample and reference arms and a spectrometer at the detector end. The spectrometer comprises of a diffraction grating (1200 grooves/mm) and a CCD line scan camera (4096 pixel resolution, 29.3 kHz line rate). The interference signal from the sample and the reference arms of the Michelson interferometer is detected by the spectrometer and digitized by an image acquisition card (NI-IMAQ PCI-1428). Depth profile (A-line) is obtained by converting the interference signal detected by the IMAQ into linear k-space [19]. The imaging axial and lateral resolution of the OCT system is about $6 \mu\text{m}$ in biological tissue.

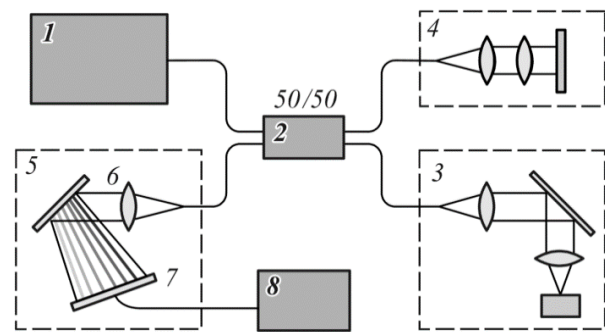


Fig. 1 Spectral domain OCT scheme: 1 – broadband source, 2 – 50/50 beam splitter, 3 – sample arm, 4 – reference arm, 5 – spectrometer with grating 6 and CCD camera 7, 8 – computer with IMAQ.

The experimental data set contains 530 OCT images with normal skin and tumors as Basal Cell Carcinoma (BCC), Malignant Melanoma (MM) and Nevus. The institutional Review Board of Samara National Research University approved the study protocol. This research adhered to the tenets set forth in the Declaration of Helsinki. Informed consent of each subject was obtained.

2.2 Haralick features

For evaluating Haralick features, a gray-level co-occurrence matrix (GLCM) from image I should be calculated. GLCM is based on frequency evaluating of a pixel with gray-level value i horizontally (vertically, diagonally) connected to a pixel with the value j . Each element (i, j) in GLCM specifies the number of times that the pixel with value i occurred horizontally adjacent to a pixel with value j [20].

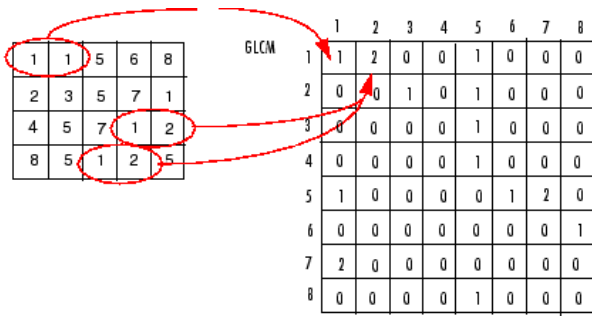


Fig. 1 A gray-level co-occurrence matrix sketch [21].

For calculating Haralick's features, image is represented as a grate of pixels of mandatory intensity, which is used for calculating four GLCMs of relative frequencies of pixels' order in directions 0° , 45° , 90° , 135° . Textural features based on these matrixes are used for images classification as will be described later.

Homogeneity returns a value that measures the closeness of the distribution of elements in the GLCM to the diagonal GLCM.

$$f_1 = \sum_{i,j} p(i,j). \quad (1)$$

Correlation serves as a measure of dependency of neighboring pixels over the whole image.

$$f_3 = \sum_{i,j} \frac{(i - \mu_i)(j - \mu_j)p(i,j)}{\sigma_i \sigma_j}. \quad (2)$$

Variance defines a measure of the intensity contrast between a pixel and its neighbor over the whole image.

$$f_4 = \sum_{i,j} |i - j|^2 p(i,j). \quad (3)$$

Energy is calculated as the sum of squared elements in the GLCM.

$$f_5 = \sum_{i,j} \frac{p(i,j)}{1 + |i - j|}. \quad (4)$$

2.3 Fractal analysis

In the analysis of OCT images, fractal analysis has been used to examine the structural change of biological tissue. For example, Fluearu [22] utilized the box counting method to compute the fractal dimension to characterize porcine arterial tissue. Sullivan [23] used the box counting method to evaluate the fractal dimension to detect the breast carcinoma. Gao [24] applied the power spectrum method to carry out the fractal analysis on the layered retinal tissue for diagnosing the diabetic retinopathy.

The most popular algorithm for computing the fractal dimension of one dimensional and two dimensional data is the box counting, method originally

developed by Voss [25]. In this method, the fractal surface is covered with a grid of n -dimensional boxes or hyper-cubes with side length, ε and counting the number of boxes that contains a part of the fractal $N(\varepsilon)$. As for signals, the grid consists of squares and for images, the grid consists of cubes. The fractal surface is covered with boxes of recursively different sizes. An input signal with N elements or an image of size $N * N$ is used as input where N is a power of 2 [26].

$$D_{bc} = \frac{\log N(\varepsilon)}{\log \frac{1}{\varepsilon}}. \quad (5)$$

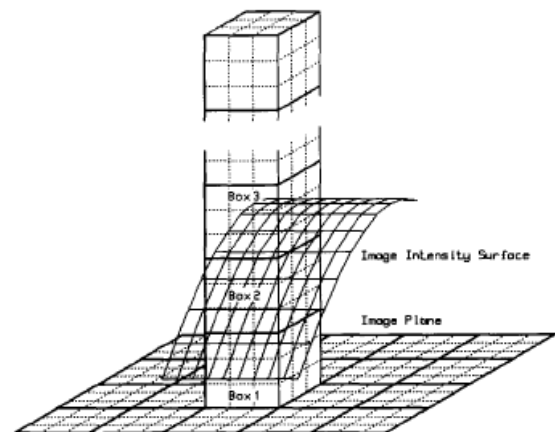
Other method for counting fractal dimension is differential box-counting method [27]. In this method, N is counted in other way. Consider that the image of size $M \times M$ pixels has been scaled down to a size $s \times s$, where $M/2 \geq s \geq 1$ and s is an integer. Then we have an estimate of $\varepsilon = s/M$. Now, as in previous techniques, consider the image as a 3-D space with (x,y) denoting 2-D position and the third coordinate (z) denoting gray level. The (x,y) space is partitioned into grids of size $s \times s$. On each grid there is a column of boxes of size $s \times s \times s'$. If the total number of gray levels is G then $G/s' = M/s$. For example, see Fig. 3, where $s = s' = 3$. Assign numbers 1, 2, ... to the boxes as shown. Let the minimum and maximum gray level of the image in the (i,j) th grid fall in box number k and l , respectively. In this approach

$$n_r(i,j) = l - k + 1 \quad (6)$$

is the contribution of N_r , in (i,j) th grid. For example, in Fig. 3, $n_r(i,j) = 3 - 1 + 1$. Taking contributions from all grids, we have

$$N_r = \sum_{i,j} n_r(i,j). \quad (7)$$

N_r is counted for different values of r , i.e., different values of s . Then using (7), it is possible to estimate D , the fractal dimension, from the least square linear fit of $\log N_r$ against $\log(1/r)$.

Fig. 3 Determination of n_r by differential box-counting method [28].

Also we used power-spectrum method for counting fractal dimension [29]. This method is an application of the Fourier power spectrum method. The real-space image is Fourier transformed by means of the fast Fourier transform and the power spectrum, P_i is computed as:

$$P_i = \text{Re}(k_i)^2 + \text{Im}(k_i)^2. \quad (8)$$

Then the power spectrum of an ideal one dimensional fractal signal with dimension D is considered. This has the formula

$$P_i = c k_i^{-\beta}, \quad (9)$$

where c is a constant and β is the spectral exponent. The index β is related to the Fourier transform dimension D_F . The value of the spectral β and D_F , can be found out for the input signal by fitting a least squares error line to the data. The merits of this approach are it is generalizable, potentially more accurate and the computation of D_F is based on an explicit formula [23].

2.4 The complex directional field

Complex directional field defines as:

$$\dot{\varphi}(x, y) = w(x, y) \exp(i2\varphi(x, y)), \quad (10)$$

where $w(x, y)$ has a physical meaning as reliability of directional field in this point [17].

Methods of local gradients are based on the fact, that function's gradient in each point is perpendicular to tangency of contour line in this point. These methods are based on evaluation of gradient intensity function for different positions of local mask inside scanned on image outer window W with size $M * N$ (local gradient) $(f_x^{k,l}, f_y^{k,l})$, where $1 \leq k \leq N, 1 \leq l \leq M$.

$$\tan \hat{\varphi}(x, y) = -\frac{f_x}{f_y}, 0 \leq \hat{\varphi}(x, y) \leq \pi, \quad (11)$$

$$(f_x, f_y) = \left(\frac{\partial I(x, y)}{\partial x}, \frac{\partial I(x, y)}{\partial y} \right).$$

The local gradient methodology is divided into two classes. First one is methods of gradient projections averaging and second one is methods of local direction angles averaging. Method of gradient projections averaging is based on using of local gradients, mandatory to position (k, l) of local mask in calculating of intensity function gradient in the center of outer window W :

$$(f_x, f_y) = \frac{1}{NM} \sum_{k=1}^N \sum_{l=1}^M (f_x^{k,l}, f_y^{k,l}). \quad (12)$$

Method of local direction angles averaging uses local gradients $(f_x^{k,l}, f_y^{k,l})$ for calculation of local angles:

$$\varphi_{k,l} = -\tan^{-1} \left(\frac{f_x^{k,l}}{f_y^{k,l}} \right). \quad (13)$$

Then direction of trace in the center of outer window W can be evaluated by averaging of local angles field:

$$\hat{\varphi} = \frac{1}{2} \arg \sum_{k=1}^N \sum_{l=1}^M \exp(i2\varphi_{k,l}). \quad (14)$$

The value of a weight function of a direction field will be next:

$$\hat{w} = \left| \frac{1}{MN} \sum_{k=1}^N \sum_{l=1}^M \exp(i2\varphi_{k,l}) \right|. \quad (15)$$

2.5 Markov random fields

A Markov random field, Markov network or undirected graphical model is a graphical model in which a set of random variables have a Markov property described by an undirected graph [30]. Connection of nodes with each other is defined by localities system as $N = \{N_s | s \in S\}$, where N_s is a neighbors set of node s , S is finite set of nodes. Localities system has property: $s \notin N_s$. Random field is called as Markov random field in relation to localities system, if for all $z_i \in Z$ next condition is realized: $P(z_i | z_{S/i}) = P(z_i | z_{N_i})$, where $Z = \{Z_1, \dots, Z_m\}$ – random field, z_i – values of random variables Z_i . We use suggestion that OCT-image is Markov Random Field, so pixel intensity depends only from intensities of neighboring pixels. Non-causal locality is used [15]. We estimate autocorrelation function as [31]:

$$R(x, y) = \sum_{\substack{i=-1 \\ I(x+i, y+j) \in N_{x,y}}}^1 \sum_{\substack{j=-1 \\ I(x+i, y+j) \in N_{x,y}}}^1 I(x, y) I(x+i, y+j). \quad (16)$$

Then we find variation and mean for using them as textural features.

2.6 Neural networks

Neural network is a mathematician model that simplifies imitated work of human brain. A standard neural network consists of many simple, connected processors called neurons, each producing a sequence of real-valued activations. Input neurons get activated through sensors perceiving the environment; other neurons get activated through weighted connections from previously active neurons. Some neurons may influence the environment by triggering actions [32]. Neuron is a base element of neural system [33]. It has two types of appendages: with input information

(dendrites) and with output information (axon, only one for translating impulse to other neurons) [34]. Neural joints between neurons (synapses) play a role of weights to accelerate or slow down impulses. If algebraic sum of impulses exceeds threshold value, then neuron translate impulse to other neurons [35]. Below you can see a mathematician explanation in figure and formulas, where $u_1, \dots, u_n$ – input signals; $w_1, \dots, w_n$ – synaptic weights; y – output signal; v – threshold value.

$$y = \begin{cases} 1, \sum_{i=1}^N w_i u_i \geq v; \\ 0, \sum_{i=1}^N w_i u_i < v; \end{cases} \quad (17)$$

$$y = f(\sum_{i=0}^N w_i u_i); \quad (18)$$

$$f(x) = \begin{cases} 1, x \geq 0 \\ 0, x < 0 \end{cases} \quad (19)$$

where $w_0 = v$; $u_0 = -1$.

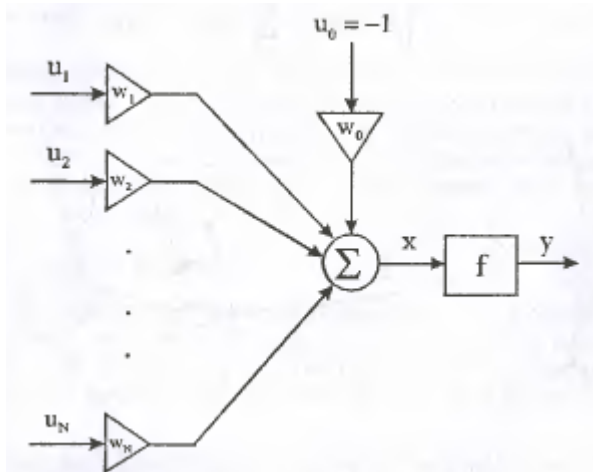


Fig. 4 Model of neuron from artificial neural network [33].

This paper uses scaled conjugate gradient backpropagation [36] based artificial neural network for tissues discrimination (tumor, norm; melanoma, nevus, basalioma or healthy skin) quality assessment. The ANN is trained in MATLAB using 10 corresponding parameters for B-scans as fractal dimensions, counted by 1D-box-counting method (with standard deviation), 2D-differential box counting method and 2D-power spectrum method, Haralick features (contrast, correlation, homogeneity, energy) and Markov random fields features (variance and mean of autocorrelation function estimation). The quantity of hidden layers is 10. For C-scans complex directional features (field variance and weight function variance) were used, the quantity of hidden layers was 20.

2.7 Boosting

Despite the possible good results of classifiers, it is always possible to improve them by linear combination. In our case we use boosting [18]. The task of precedents learning is considered. $\langle X, Y, y^*, X^l \rangle$, where X – space of

objects; $y^*: X \rightarrow Y$ – unknown target dependence; $X^l = (x_1, \dots, x_l)$ – is a learning selection; $Y^l = (y_1, \dots, y_l)$ is a vector of answers on learning objects $y_i = y^*(x_i)$. It is needed to build an algorithm: $X \rightarrow Y$, that approximated target dependence y^* on all set X [37].

The task of classification on two classes is considered, $Y^l = \{-1, +1\}$. We suggest that solution rule is fixed, $C(b) = \text{sign}(b)$.

Base algorithms return answers -1, 0, +1. Answer $b_t(x) = 0$ means that base algorithm b_t refused from classification object x and answer $b_t(x)$ don't use in composition. Finding algorithm composition has look [38]:

$$\begin{aligned} a(x) &= C(F(b_1(x), \dots, b_T(x))) = \\ &= \text{sign}(\sum_{t=1}^T a_t b_t(x)), x \in X \end{aligned} \quad (20)$$

This paper uses conjugate AdaBoost [5] algorithm for tissues discrimination (tumor, norma; MM, nevus, BCC or healthy skin) quality assessment. The boosting for B-scans uses 10 corresponding parameters as fractal dimensions (FD), counted by 1D-box-counting method (with standard deviation (SD)), 2D-differential box counting method and 2D-power spectrum method, Haralick features (contrast, correlation, homogeneity, energy) and Markov random fields features (variance and mean of autocorrelation function estimation). For C-scans complex directional features (field variance and weight function variance) are used, the maximum quantity of trees [39] was 200.

3 Results and Discussion

The anisotropic algorithm has been used for denoising. Then images are processed by four methods: Haralick's features, fractal analysis, Markov random fields features (for B-scans) and complex directional field features (for C-scans).

A CCD-sensor in camera is one of noise sources in any acquisition system. The final denoised B-scan and original are shown on Fig. 5. One can see background, edge of skin and inner layers with characterized dark cores ("nests") of BCC. After filtration, the quality of image is visually improved. Moreover, the Signal-to-Noise Ratio (SNR) increases 1.4 times in heterogeneous regions to 4 times in background and homogeneous regions.

The Fig. 6 shows a separating healthy skin from MM. The Fisher's linear discriminant analysis has been used in all cases described here [40] and classes are good separated by the linear classificatory. The MM–Skin separating achieved 88% sensitivity and 92.8% specificity for the contrast-correlation method. In case of correlation-homogeneity and MM–nevus, 88% sensitivity for linear classificatory, also as 95.2% specificity have been obtained.

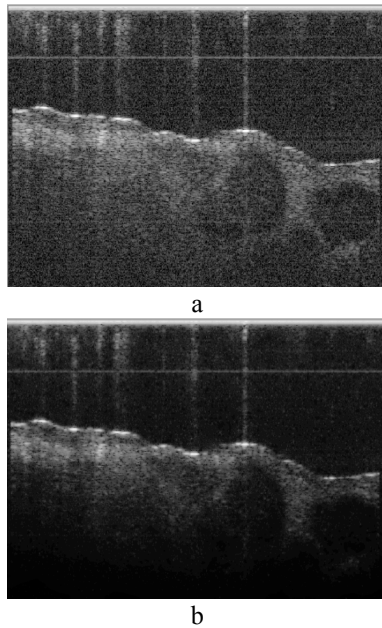


Fig. 5 (a) Original B-scan of BCC and (b) B-scan proceeded after filtration.

The Fig. 7(a) shows a case healthy skin versus MM. The sensitivity for variance of AF (MRF) –mean of AF (MRF) is 92.8%, specificity is 95.2%. On the Fig. 7(b) for MM–BCC we have 90.4% and 83.3% respectively by using MRF features. One can observe a stronger

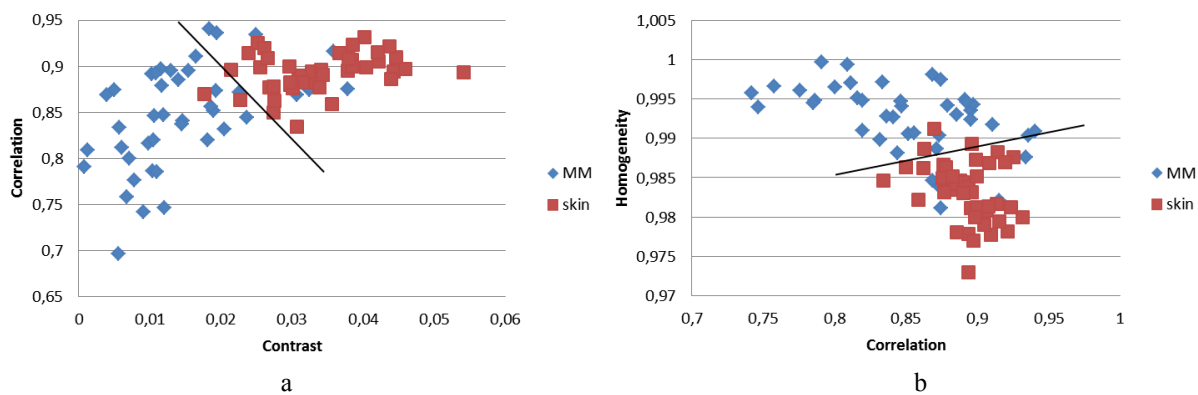


Fig. 6 (a) Contrast–Correlation for MM–Healthy Skin and (b) Correlation–Homogeneity for MM–Healthy Skin.

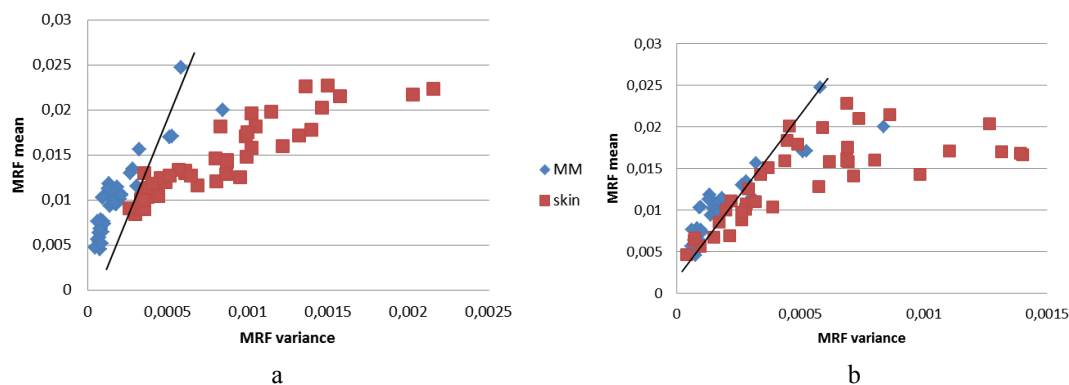


Fig. 7 (a) Variance MRF–Mean MRF for MM–Healthy Skin and (b) variance MRF–Mean MRF for MM–BCC.

correlation in this case between features in contrast with Fig. 6. Generally, that means this feature's pair could be reduced to one single feature. But, in the case, an angle between the all data approximation (the “trend”) line and Fishers' discriminant one is less than $\pi/2$, which means both features are particularly valuable.

The Fig. 8 shows us C-scan of skin, the complex directional field of image and weight function. Optical tissue properties changing leads to modification of “sand” texture features can be detected by naked eye also. The Fig. 9 shows dividing MM from BCC and BCC from Nevus. For MM–BCC sensitivity is 91.5%, specificity is 100%. For BCC–Nevus sensitivity is 100%, specificity is 97.5%. This case is also demonstrating a very strong correlation, but, this time, this is an inner class correlation. Both classes are good correlated and, which is much more important, discriminated. This is not correct for small mixed groups on right side of both parts of the Fig. 9. That fact may be explained by a fact MM and BCC tissues could have a very complex topology and a not trivial histology report. In many cases the MM is growing inside a nevus background. Moreover, the MM/BCC could be mixed with other benign tumors. But in our study we choose a binary classes system with no details.

The Fig. 10(a) shows dividing norm from tumor with sensitivity in 86.9% and specificity in 89.3% after

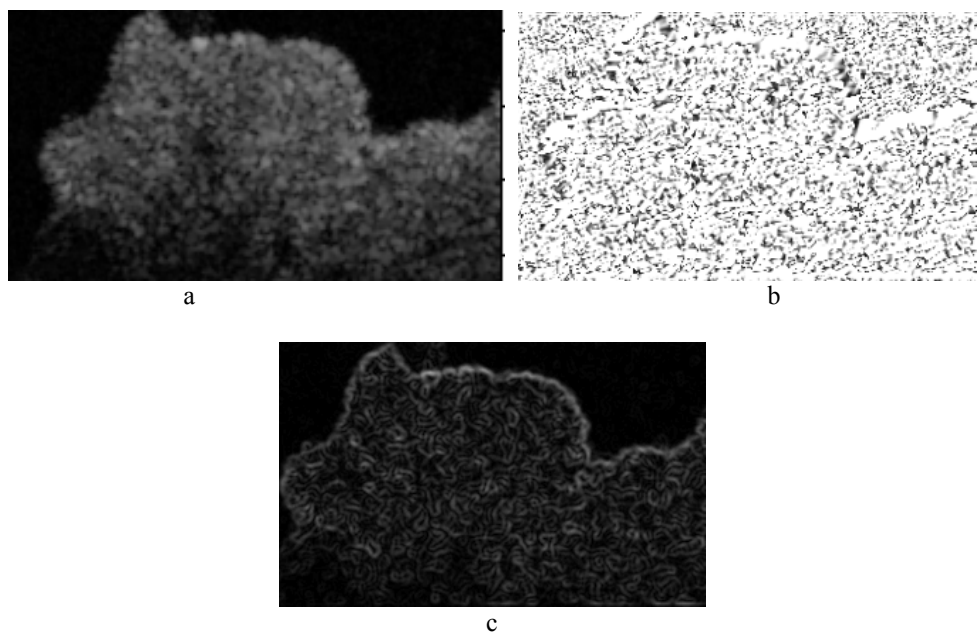


Fig. 8 (a) Original image, (b) complex directional field and (c) weight function [41, 42].

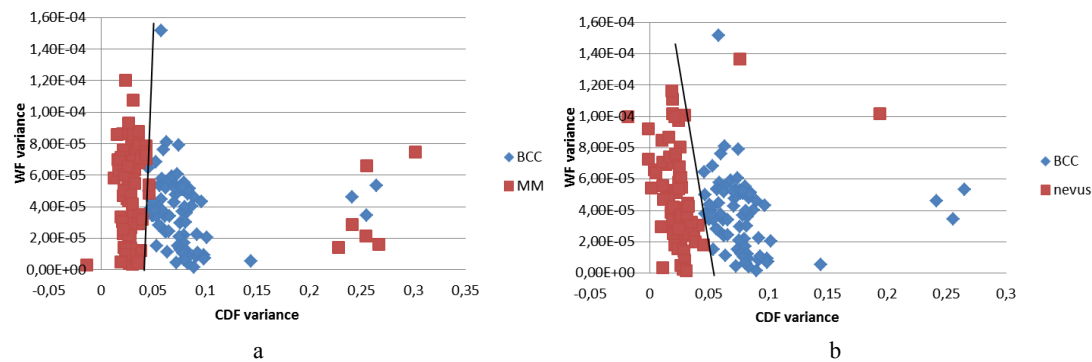


Fig. 9 (a) Complex directional field variance – weight function variance for MM–BCC and (b) complex directional field variance – weight function variance for BCC – nevus.

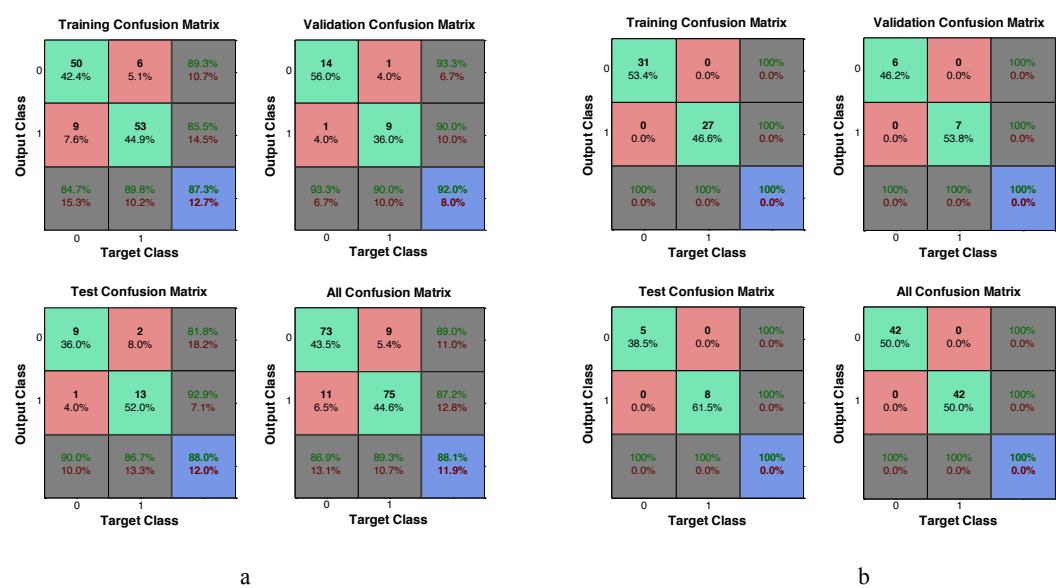


Fig. 10 (a) Confusion matrixes for Tumor–Norm and (b) MM–Healthy Skin.

using neural network with scaled conjugate gradient backpropagation for pattern recognition. The Fig. 10(b) shows dividing MM from healthy skin with sensitivity 100% and specificity 100%. This incredible result can be explained by a little dataset for ANN in this case, so it should be checked in bigger sets in future investigations

The Fig. 11(a) shows dividing tumor from norm with sensitivity in 73.8% and specificity in 85% after

using neural network with scaled conjugate gradient backpropagation for pattern recognition. The Fig. 11(b) shows classification of species between four classes (MM, BCC, Nevus and Healthy Skin) with precision more than 75% by using boosting. The summary is presented in Table 1. This amplification is linked with a composition of all features' information, received from tumors and healthy skin on OCT images.

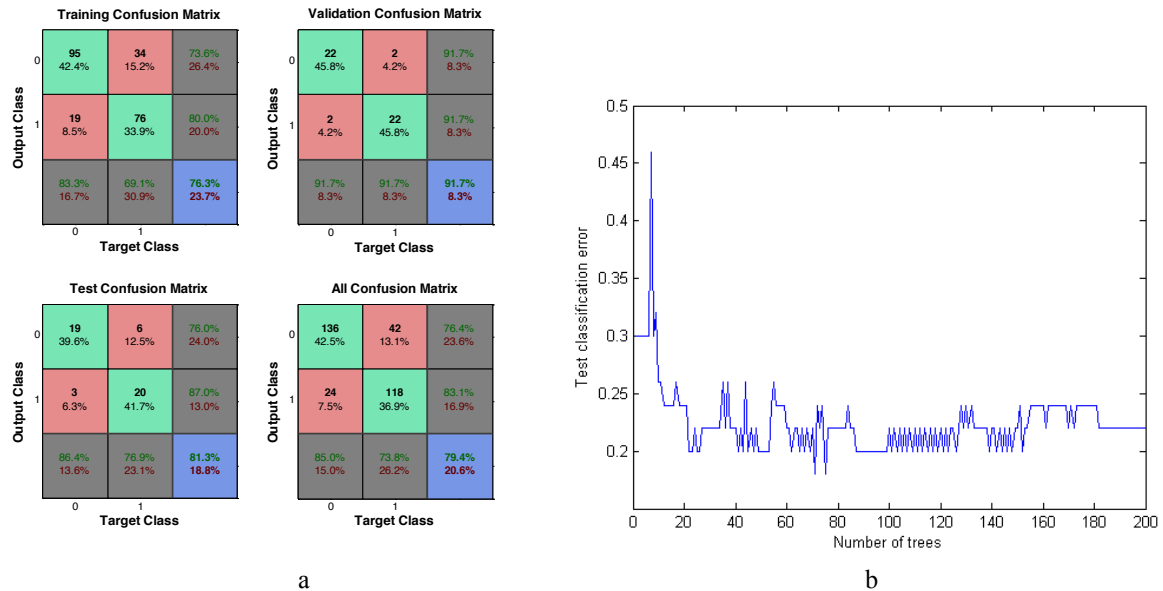


Fig. 11 (a) Confusion Matrix for Norma-Tumor and (b) boosting results for Tumor-Norma case.

Table 1 Statistical characteristics of separation tissues by utilized features.

Category of feature	Name of feature	Tissues		Precision	Quantity	Type
		Tissue1	Tissue2	(sensitivity-specificity)	(images)	(B/C-scan)
Haralick	Contrast-Correlation	MM	Healthy Skin	88%-92,8%	42/42(84)	B
Haralick	Correlation-Homogeneity	MM	Healthy Skin	88%-95,2%	42/42(84)	B
MRF	AF variance-AF mean	MM	Healthy Skin	92,8%-95,2%	42/42(84)	B
MRF	AF variance-AF mean	MM	BCC	90,4%-83,3%	42/42(84)	B
CDF	WF variance-CDF variance	Nevus	Healthy Skin	97,5%-83,7%	80/80(160)	C
CDF	WF variance-CDF variance	BCC	Nevus	100%-97,5%	80/80(160)	C
CDF	WF variance-CDF variance	MM	BCC	91,5%-100%	80/80(160)	C
Neural networks	Scaled conjugate gradient backpropagation	Tumor	Norma	86,9%-89,3%	84/84(168)	B
Neural networks	Scaled conjugate gradient backpropagation	Tumor	Norma	73,8%-85%	160/160(320)	C
Fractal	SD-FD	Tumor	Norma	70%-71%	42/84(126)	B
Neural networks	Scaled conjugate gradient backpropagation	MM	BCC	100%-100%	42/42(84)	B
Boosting	AdaBoost	MM, BCC	Nevus, Healthy Skin	75%	42/42/42/42(168)	B

So after that we can say that all methodologies show good results in classifying melanoma from healthy skin. Boosting and neural networks improve significantly all results for other classifications cases. If we compare boosting and neural networks, then we reveal that ANN shows sensitivity and specificity higher than boosting on same set of B-scans. But boosting let us to divide between four classes (MM, BCC, nevus and healthy skin), but not between 2 classes as other classifiers (for example, Tumor (BCC + MM) and Norma (Nevus + Healthy Skin)). So we receive more universal classifier that can be used with high precision without waiting histological data for diagnostics.

For comparing our results with other works, we should say few words about achievements of other researchers. Gambichler et al. [43] received sensitivity of about 75% and specificity of about 93% for MM and nevus in the skin tissue. Multipath OCT system [44] has been successfully used to detect basal cell carcinoma with a sensitivity of 96% and specificity of 75%. Combined method using OCT and backscatter Raman spectroscopy, characterized in 89-100% sensitivity and 93-96% specificity for OCT imaging of skin cancer [45]. Texture analysis and pattern recognition applied to dermoscopy images of malignant melanoma in [46] with a sensitivity of 89% and specificity of 93%. We also have precision on level more 90% for some cases, so that is comparable with simplified investigations. Our previous results [41, 42] are improved by creation of new more universal classifier, based on boosting and new results for CDF with better precision.

As for diversification of proposed results, utilized features not only give us information about texture of skin and tumor, but indirectly provide geometric characteristics, that can be used by physicians for ABCDE criteria. For example, fractal features help us to evaluate the “B”, which means irregularity of lesion’s border, complex directional field features in potential could show us the “E”, evolving of tumor on series of images with different dates. Markov random fields features and Haralick’s features also have probabilistic properties. Implication of different features with

different heterogeneous nature let us speak about multimodality of method, that can be amplified by features of statistics analysis technics.

4 Conclusion

This research was dedicated to differentiation between tumors and healthy skin on OCT images, using multimodal approach for cancer recognition by evaluation of some textural features. We built universal classifier with 75% precision for differentiation between four classes (MM, BCC, nevus and healthy skin). The high precision of discrimination between MM and BCC by three methods separately very good corresponds to possibility of visual diagnostics by physicians on OCT images due to a specific form of BCC neoplasm. Also we could note about good results in differentiation between BCC and nevus, MM and healthy skin. The good results by CDF and MRF are very promising to be tested for new cases and new bigger sets of OCT images. The results for MM versus nevus case that were received in our previous work [41] were decreased.

After increasing set of images the best results are on level 93.8% for sensitivity and 73.8% for specificity with using MRF. Fractal dimensions, Haralick’s and CDF features occurred not effective for this task, neural networks should be used on bigger quantity of images. So we have got to continue our investigations for solving this important challenge. For next investigation new categories of features (morphological, geometrical, statistical, textural etc.) and closer connection with ABCDE criteria (new features for asymmetry, borders irregularity, diameter, evolution) on 2D (C-scans, mostly) and 3D OCT-images should be involved.

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Laser effect on paralimbal and trabecular zones of the eye enchases hydraulic conductivity of the sclera toward normalization of the intraocular pressure

Olga Baum¹, Georgy Zheltov², Andrej Bolshunov³, Aleksey Yuzhakov¹, Alexander Omelchenko¹, Maria Obrezkova⁴, and Emil Sobol¹

¹ Institute of Photonic Technologies, Federal Scientific Research Centre "Crystallography and Photonics" of the Russian Academy of Sciences, Moscow, Russia

² B.I. Stepanov Institute of Physics, National Academy of Sciences of Belarus, Minsk, Belarus

³ State Research Institute of Eye Diseases, Moscow, Russia

⁴ Department of Chemistry, Moscow State University, Moscow, Russia

* e-mail: baumolga@gmail.com

Abstract. This paper presents the concepts and theoretical basics of the laser activation in drainage system function of eye sclera through laser effect on both paralimbal and trabecular zones including both known tracts of the intraocular fluid outflow from the eye. To address the problem of laser procedure security and consistency of obtained result, the development processes of stationary and non-stationary temperature fields and thermal stresses were studied and leading to increase in eye tissue hydraulic conductivity and providing normalization of intraocular pressure at wavelength of 1.56 μm and 534 nm. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: laser; porous system; hydraulic conductivity; intraocular pressure; paralimbal and trabecular zones.

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1 Introduction

Among the most dangerous ophthalmic diseases resulting in hypovision and visual disability, the leading position is taken by glaucoma that is a complex of pathological processes causing system disorder of intraocular pressure (IOP) stabilization [1]. In addition to study of pathogenesis and development mechanisms of this disease, development of modern technologies, IOP normalization and stabilization techniques are certainly relevant.

The aim of this paper is to present a complex of theoretical and experimental basics studies of the laser activation in drainage system function of human eye and practical recommendations on parameter optimization of the laser radiation in practice of glaucoma treatment through laser effect on paralimbal and trabecular zones.

Primary open-angle glaucoma (POAG) is the most frequently kind of glaucoma in adults at which IOP increasing is related to fluid outflow deterioration in eye drainage system because of locked Schlemm's canal and dystrophic changes both in trabecula and in intrascleral canals system.

Laser was used to treat glaucoma for the first time in 1973 [2]. Laser trabeculoplasty and laser selective trabeculoplasty are in common practice now [3–6], but it can lead to irreversible damage of Schlemm's canal.

It is known that along with mass transfer of intraocular fluid (IOF) through Schlemm's canal and eye vascular system, there is the second natural mechanism – uveoscleral [7, 8] through which up to 50% of IOF is flowed in children, but less than 3% in adults.

Eye is a closed system and effect on one of its part always leads to response in other parts. Thus, laser effect on paralimbal eye zone (in pars plana projection) can lead to changes in trabecular system and vice versa.

It is known that under effect of heterogeneous laser radiation in intensity on biological tissues there is their local heating and thermal expansion suppressed by less heated zones. As a result there are mechanical pressure and structural changes in tissue matrix [9, 10].

Thermomechanical pressure in specific ranges of amplitude and frequency has a positive effect on biological tissues cells and contributes to perform additional porous structure leading to hydraulic conductivity recovery of cartilaginous tissues that provided the basis for new technology of IOP normalization through increasing in sclera hydraulic conductivity due to transscleral thermomechanical effect of repetitively-pulsed laser radiation [11].

Depending on laser pulse duration and power, the interval between pulses and total time of radiation, the different interaction mechanisms of laser radiation and biological tissues are possible to implement.

So, at such short pulses the laser radiation can cause acoustic wave generation [12] and this process can be described by equation of medium motion in Euler and Lagrange form [13]. Euler equation and variation calculation was used to build a model of the laser-induced pressure relaxation in the cartilaginous tissues

because of pore formation [14]. In some cases, Lagrange form is more preferable and it can describe heterogeneous mediums motion whose properties are changed while transferring through the interface surface [15].

If there is a sufficient laser pulse duration, the processes of local expansion and compression lead to local thermomechanical pressures in medium and this process can be described by using a problem of the constrained vibrations of viscoelastic medium excited in volume by periodic force of water laser-heating [16].

Presented study covers both tracts of the intraocular fluid outflow from eyes (uveoscleral and trabecular) and a wide range of laser pulse duration including both non-stationary and stationary processes of pore formation and structural change under effect of laser radiation.

The first part provides a study of laser effect on paralimbal and trabecular sclera zones at wave length of 1.56 μm and an effect model by respectively long laser pulses is considered. The second part provides parameter optimization of laser radiation for functions stimulation technology of the eye drainage system by method of selective laser trabeculoplasty (SLT) at wave length of 534 nm. Theoretical models were developed for both cases and they describe laser pulses effect with a different duration on eye sclera. A series of experiments on laser effect of both zones in selected laser modes was performed.

2 Materials and methods

In this investigation two limiting cases have been considered: long pulses (~200 ms) and short pulses (from 10^{-8} to 10^{-5} s).

In the theoretical part of the study, the following methods have been used: Temperature fields in the tissue in the course of laser irradiation have been found on the basis of a solution of the thermo-conductivity problem. Computer simulations have been performed using the finite difference method in Mathematica 5.0. The thermo-mechanical effect of pulse-periodic laser radiation for long pulses has been calculated using the theory of stationary thermo-elasticity [17].

The effect of short laser pulses and the photo-acoustic response of the tissue have been calculated using the set of nonlinear differential equations, including heat conductivity equation, Navier-Stokes equation and continuity equation [18, 19]. Computer simulations in Section 3.3 have been performed using Gear method.

Experiments were performed ex-vivo on paralimbal zone of 6 eye sclera of 3 mini-pigs, Svetlogorsk population (breeding of Research Center for Biomedical Technologies at Russian Academy of Medical Sciences).

Irradiation of samples has been performed using 1.56 μm fiber laser (IPG Photonics, Inc.). IOP measurements have been performed using the Schiotz tonometer contacted with cornea of full eye. The eyes were hold fixed on the instrumental table providing the ability to perform controlled eye compression across the

equator in order to increase IOP level up to defined value. Light propagation measurements for intact and irradiated parts of sclera depending on relative position of the laser application areas both in 6 samples of trabecular and 6 samples of paralimbal zones, have been performed by previously constructed and described in the paper [20] laboratory setup. This setup included the fiber optics system that enabled to irradiate a sample perpendicular to its surface and to detect passed signal through fixed sample during irradiation. Hydraulic conductivity measurement of these samples around the laser applications were carried out by previously constructed laboratory setup described in the paper [21].

3 Theoretical modeling of thermal and thermo-mechanical effects of laser irradiation on sclera.

3.1 Thermal effect of the long laser radiation pulses

To solve the problem of laser procedure security and consistency of obtained result, first of all it is necessary to study theoretically processes of stationary and non-stationary thermal pressures leading to increase in eye tissue hydraulic conductivity and providing IOP normalization.

Previously, the laser radiation modes were defined at which the hydraulic conductivity of irradiated eye sclera increased in several times that was associated with new microscope formation, invisible using optical microscope, whose being was confirmed later as a study result of the clear fine structure changing on selected eyes [22; 23].

Theoretical study on dynamics modeling of temperature fields and thermal pressures in the paralimbal zones of eye sclera leading to microporous structure formation to provide effective IOF transport and IOP normalization was performed.

Heat conductivity equation was solved [24]:

$$\frac{\partial T(x, y, z, \tau)}{\partial \tau} = a \Delta T(x, y, z, \tau) + G(x, y, z, \tau), \quad (1)$$

where a is temperature conductivity coefficient. $G(x, y, z, \tau)$ is a volume heat source generated by laser radiation and damping with a depth by law of Beer–Lambert–Bouguer with effective absorption index κ :

$$G(x, y, z, \tau) = P(\tau) \exp\left(-\frac{y^2 + z^2}{r_0^2(x)}\right) \frac{\kappa \exp(-\kappa x)}{c \rho}. \quad (2)$$

Here $P(\tau)$ is time-dependant laser power density, c is specific heat, ρ is density.

Density of the energy incident flux to lateral surface of biological tissue has a spatial distribution corresponding to Gaussian distribution with effective

beam radius $r_0(x)$ considering beam divergence in cross direction while transferring through the mediums in x direction.

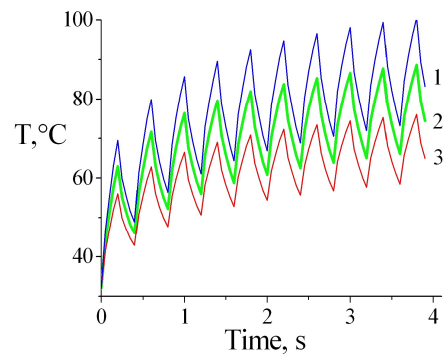


Fig. 1 The theoretically simulated maximum temperature dynamics at laser effect with power of (1) 1.1 W, (2) 0.9 W and (3) 0.7 W for the same time mode: pulse duration is 200 ms, pulse repetition frequency is 2.5 Hz, and radiation time is 4 seconds.

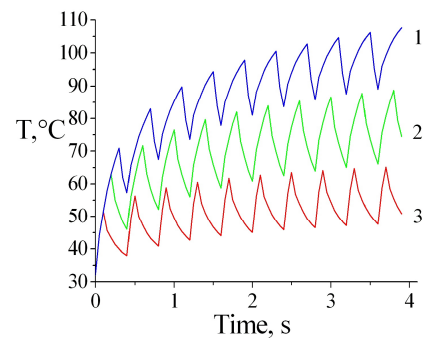


Fig. 2 The theoretically simulated maximum temperature dynamics at laser effect with power of 0.9 W for time modes with pulse duration of (1) 300 ms, (2) 200 ms and (3) 100 ms, pulse repetition frequency is 2.5 Hz, and radiation time is 4 seconds.

The previously selected range of laser modes [25] was studied for wavelength of 1.56 μm . Fig. 1 presents numerical simulation of the maximum temperature on the base of the equation (1) and (2). Time variations of maximum temperature at laser radiation are shown for different power values at pulse duration of 200 ms, pulse repetition frequency of 2.5 Hz, and radiation time of 4 seconds. Total time of being higher 70°C for power of 0.7 W is 0.5 seconds, for power of 0.9 W is 2 seconds and for power of 1.1 W is 2.8 seconds, but hereby the fair average temperature which enables to maintain heating about 70°C was obtained at radiation with power of 0.9 W; there was “underheating” at that radiation power of less than 0.8 W and there was obvious overheating at power of more than 1 W causing denaturation.

Modeling of the effect modes with different laser pulse duration was performed for selected average power of 0.9 W. Fig. 2 shows the time variations of maximum temperature at laser radiation with power of

0.9 W for pulse duration of 300 ms (curve 1), 200 ms (curve 2) and 100 ms (curve 3). Total time of being higher 70°C at pulse duration of 300 ms was 3.45 s, for 200 ms was 2 s and for 100 ms a curve was below 70°C during the whole radiation. The fair average temperature which enables to maintain heating about 70°C was obtained only at pulse duration of 200 ms when pulse repetition frequency was 2.5 Hz. Consequently, the theoretical modeling enables to predict a range of laser parameters in which the desired result of laser effect can be expected.

Based on provided model, we can find an optimal range of the laser modes by varying the laser effect parameters to achieve the desired change of sclera pore structure under effect on its paralimbal part.

3.2 Thermo-mechanical effect of the long laser pulses

The theoretical modeling of thermal pressure field generation in the sclera paralimbal zone while surface laser radiation [24] was made on the basis of [17]. In case of the contact surface sclera heating by laser fiber optics, the bidimensional problem of thermo elasticity for thermal field occurring in plate during the process of laser heating was considered and it has the following analytical solution for radial and angular component of thermal pressures:

$$\begin{aligned}\sigma_r &= \alpha E \left(\frac{1}{b^2} \int_0^b T(r) r dr - \frac{1}{r^2} \int_0^r T(r) r dr \right); \\ \sigma_\theta &= \alpha E \left(-T(r) + \frac{1}{b^2} \int_0^b T(r) r dr + \frac{1}{r^2} \int_0^r T(r) r dr \right),\end{aligned}\quad (3)$$

where b is boundary of integration range within radius r , α is thermal expansion coefficient, E is elasticity modulus.

Plastic strain range is defined by Mises/Tresca-Saint-Venant criterion: $\sigma_\theta - \sigma_r = \sigma_s$, where σ_s is pressure corresponding to yield point [26]. Specifically, the plastic strain range is an epicenter around which there are maximum pressures causing pore formation as evidenced by experimental data given in the experimental part of this article (section 4).

3.3 Thermo-mechanical effect of the short laser pulses

Effect of short laser radiation pulses is used in the method of SLT, whereby a special gonioscopy lens (Goldman's lens) focus a laser fluxon the internal surface of trabecula and apply sequences of applications. Mainly, the modern SLT version uses pulse radiation of green spectral range with short pulse duration of ns order [27].

The object of this section is to perform the dependence analysis of heating dynamics at wave length

of 534 μm and parameters of generated acoustic wave as well as potential zone topography of the cavitation tissue destruction from the radiation wave length during variation of laser pulse duration from 10^{-8} to 10^{-5} s. The parameters for numeric simulation were taken from [28].

Trabecula matrix is transparent in the visible spectral range, so laser radiation is selectively absorbed by melanin contained in pigmented granulas. Flash granules heating is close in nature to isochoric one (constant-volume heating). It followed by field formation of the internal pressures and subsequent generation of acoustic waves. Acoustic waves formed by specific granules are summarized and exerts heavy and oscillatory mechanical effect on trabecular plates. Such effect cleans both gaps between plates and capillary holes in plates themselves. Furthermore, this original "massage" stimulates and reactivates metabolic processes in the trabecula matrix.

In view of given above information for parameters calculation of the photoacoustic response of trabecula tissue on effect of the short laser radiation pulses, the following physical model is considered:

(1) unpigmented trabecula matrix is modeled by homogeneous medium, whose physical, thermal-physical, optico-spectral properties are appropriate water properties.

(2) It is expected that pigment granules have spherical shape. Characteristic dimensions (diameters) of these particles have Gaussian (normal) distribution with a half-width in the interval 10 to 50 μm on exp(-1) level.

(3) Absorption spectral index $k(\lambda)$, [m^{-1}] of the pigment granules is defined by melanin contained in their composition. To evaluate the absolute values $k(\lambda)$, we use data given in [29, 30] for similar light-absorbing particles (melan-protein granules – MPG) contained in pigmental epithelium of eye retina. Values of absorption spectral indexes $k(\lambda)$ for radiation with wave length of 400, 500 and 600 is equal to $6 \cdot 9 \times 10^5$, 3.3×10^5 and 1.9×10^5 , [m^{-1}] correspondingly.

(4) Laser beam diameter exceeds the spherical model diameter of pigment granule by several orders of magnitude. For this reason, the power-density distribution of radiation within aperture defined by absorptive particle diameter can be considered as uniform.

(5) At given values of absorption spectral indexes, a radiation incoming on medium is almost completely absorbed in a layer with thickness of μm unity order.

(6) With reference to 3, 4, 5 item, an average volume density of optic radiation Q (W/m^3) absorbed by spherical granule of radius R , ($10 \leq R \leq 50 \mu\text{m}$) can be calculated using the following formula:

$$Q = E \frac{S}{V} = E \frac{\pi R^2}{\frac{4}{3} \pi R^3} = 0.75 \frac{E}{R}, \quad (4)$$

where $S(m^2)$ is input aperture area of radiation flux, $V(m^3)$ is spherical particle volume, $E [W/m^2]$ is particle surface irradiation.

(7) Q value does not depend on radiation wave length in the spectral interval of 400-600 nm.

In considered approximating, the radiation time (laser pulse duration) is 2-3 order less than thermal relaxation time of considered spherical light-absorbing particles in medium with thermal-physical water properties. For these conditions, medium state in two-dimensional axially-symmetrical approximation is described by the following set of nonlinear differential equation (heat conductivity equation, Navier-Stokes equation, continuity equation) [18, 19]:

$$\begin{aligned} \frac{dT}{dt} &= Q + \frac{1}{\rho c_p} \frac{dP}{dt} + \eta \nabla^2 T; \\ Q &= \frac{E_0 \cdot (k+s)}{\rho c_p} \exp\left\{-(k+s)z - (r/r_0)^2\right\}; \\ \frac{du}{dt} &= -\frac{1}{\rho} \frac{\partial P}{\partial z} + \mu \nabla^2 u; \\ \frac{dv}{dt} &= -\frac{1}{\rho} \frac{\partial P}{\partial r} + \mu \left(\nabla^2 v - \frac{v}{r^2} \right); \\ \frac{d\rho}{dt} &= -\rho \left(\frac{\partial u}{\partial z} + \frac{1}{r} \frac{\partial}{\partial r} r v \right) = -\rho \cdot \text{div} \left(\vec{v} \right), \end{aligned} \quad (5)$$

where

$$\begin{aligned} \frac{d}{dt} &= \frac{\partial}{\partial t} + u \frac{\partial}{\partial z} + v \frac{\partial}{\partial r}; \\ \nabla^2 &= \frac{\partial^2}{\partial z^2} + \frac{1}{r} \frac{\partial}{\partial r} r \frac{\partial}{\partial r}; \\ \text{div} \left(\vec{v} \right) &= \frac{\partial u}{\partial z} + \frac{1}{r} \frac{\partial}{\partial r} r v, \end{aligned}$$

r, z, t are radial, axial and time coordinates;

T is temperature, P is pressure, u, v are axial and radial local speeds; c_p is heat capacity at constant pressure, ρ is density, η is temperature conductivity coefficient, μ is kinematic coefficient of viscosity. For water at temperature of 310°K:

$$\begin{aligned} \rho &= 10^3 \frac{kg}{m^3}, \\ c_p &\approx 4.2 \cdot 10^3 \frac{J}{kg \cdot K}, \\ \eta &= 0.14 \cdot 10^{-6} \frac{m^2}{s} (P=10^5 \text{ Pa}). \end{aligned}$$

Set of equation (5) should be supplemented by state equation $\rho = \rho(P, T)$.

We transform it by the following thermodynamic relations:

$$\rho = \rho(P, T); \quad \delta\rho = \frac{\partial\rho}{\partial P} \delta P + \frac{\partial\rho}{\partial T} \delta T;$$

$$\frac{\partial\rho}{\partial P} = -\frac{1}{V^2} \frac{\partial V}{\partial P} = \alpha\rho; \quad \frac{\partial\rho}{\partial T} = -\frac{1}{V^2} \frac{\partial V}{\partial T} = -\beta\rho;$$

$$\alpha = \frac{1}{V} \frac{\partial V}{\partial P} - \text{adiabatic compressibility}, \quad (6)$$

$$\beta = \frac{1}{V} \frac{\partial V}{\partial T} - \text{volume coefficient of thermal extension},$$

$$\alpha \approx 4.4 \cdot 10^{-10} \text{ Pa}^{-1} (\text{H}_2\text{O}, P=10^5 \text{ Pa} \quad T=310, ^\circ \text{K}),$$

$$\beta \approx 0.4 \cdot 10^{-3} \text{ K}^{-1} (\text{H}_2\text{O}, P=10^5 \text{ Pa} \quad T=310, ^\circ \text{K}).$$

Taking into account relations (6), the fourth equation in set (5) is written as:

$$\alpha \frac{dP}{dt} - \beta \frac{dT}{dt} = -\text{div} \left(\vec{v} \right) \quad (7)$$

$T(r, z, t), P(r, z, t), u(r, z, t), v(r, z, t)$ dependences are defined as a result of the numerical calculation of equation set (5)-(7).

With respect to specific situation, considered in this paper (spherical absorptive particles, homogeneous transparent medium), the set of equations (5)-(7) can be reduced to one-dimensional case (radial symmetry) and thereby significantly simplified. Transition sequence to one-dimensional problem is considered in [31]. Set of equation (5) is written as:

$$\begin{aligned} \frac{dT}{dt} &= Q + \frac{1}{\rho c_p} \frac{dP}{dt} + \eta \frac{1}{x^{g-1}} \frac{\partial}{\partial x} x^{g-1} \frac{\partial T}{\partial x}; \\ \frac{dU}{dt} &= -\frac{1}{\rho} \frac{\partial P}{\partial x} + \mu \left(\frac{1}{x^{g-1}} \frac{\partial}{\partial x} x^{g-1} \frac{\partial U}{\partial x} - (g-1) \frac{U}{x^2} \right); \\ \frac{d\rho}{dt} &= -\rho \left(\frac{1}{x^{g-1}} \frac{\partial}{\partial x} x^{g-1} U \right), \quad \text{where } \frac{d}{dt} = \frac{\partial}{\partial t} + u \frac{\partial}{\partial x}. \end{aligned} \quad (8)$$

Here x is equivalent to r , g is parameter that is equal to 3 for spherical symmetry.

For Gaussian distribution

$$Q(t, r) = \frac{E_0(t) \cdot k}{\rho c_p} \exp\left\{-(r/r_0)^2\right\}. \quad (9)$$

Having combined continuity equation and state equation similarly to (6), (7) the last equation in set (8) can be written as:

$$\alpha \frac{dP}{dt} - \beta \frac{dT}{dt} = -\frac{1}{x^{g-1}} \frac{\partial}{\partial x} x^{g-1} U. \quad (10)$$

Boundary conditions on free boundary:

$$P|_B = P_0, \quad \frac{\partial T}{\partial x}|_B = 0, \quad \frac{\partial U}{\partial x}|_B = 0. \quad (11)$$

Symmetry condition is set additionally for cases of spherical symmetry when $r=0$:

$$\left. \frac{\partial P}{\partial x} \right|_0 = \left. \frac{\partial T}{\partial x} \right|_0 = 0, \quad U|_0 = 0. \quad (12)$$

Space along the r coordinate is divided into N layers for numerical calculation. The required functions are solution of $3N$ differential equation set using Gear method. A range of solution results of (8)-(10) equation set is shown on Fig. 3.

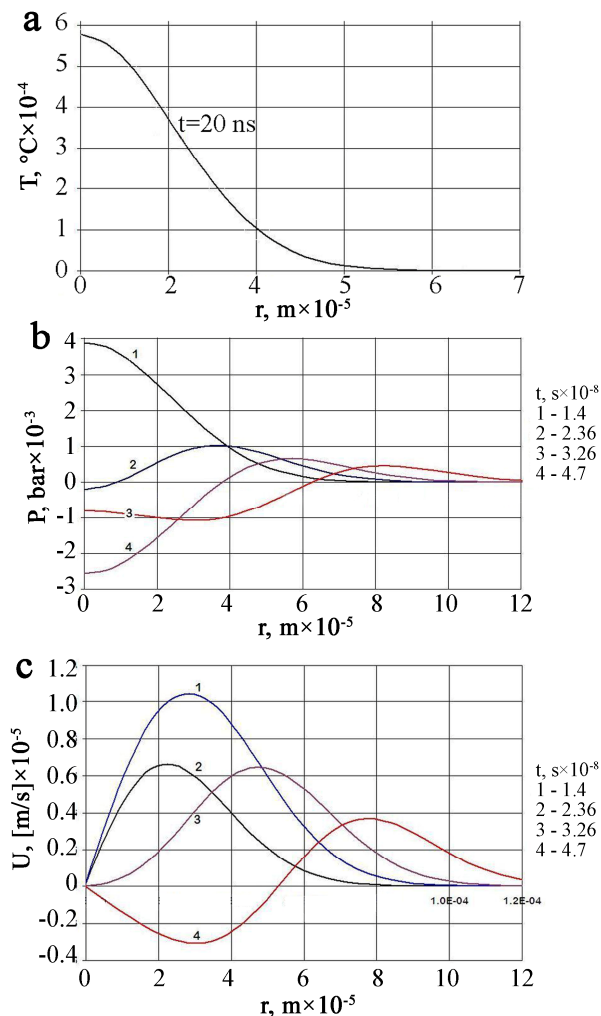


Fig. 3 (a) The theoretically simulated temperature distribution T along radial coordinates as a result of spherical light-absorbing particles with a diameter of $60 \mu\text{m}$ by laser pulse with a width of 20 ns ; (b) the theoretically simulated sequence of acoustic wave formation for conditions related to (a); (c) the theoretically simulated radial speeds evolution of medium particles for conditions related to (a) and (c).

Fig. 3 show the formation dynamics of temperature fields, pressure (acoustic wave) and radial speed of medium particles under triangle pulse effect of the laser radiation of visible spectral range (in interval $400\text{--}600 \text{ nm}$) with duration of 20 ns around the basis on spherical

model of pigment granule with a diameter of $60 \mu\text{m}$. Medium irradiation E is normalized to 1 kW/m^2 value.

In accepted approximations to the pulse termination time, granule heating approaches to maximum at the sphere center (Fig. 3(a)). Normal temperature distribution approaches to Gaussian distribution involving border zones of unpigmented matrix with a granule. Temperature gradient reaches the maximum value to about 14-th ns from radiation beginning. Speed of medium thermal expansion is finite and in our conditions it does not exceed sound speed. As heating rate is extremely high, the conditions similar to isobaric one are formed in the central part of granule (constant-volume heating). At that, there is a pressure field of volumetric compression in the central area (positive branch of pressure amplitude divergence; Fig. 3(b), curve 1). Natural response of medium is an adequate speed growth of the medium thermal expansion (particles motion from center to periphery; curve 1 in Fig. 3(c)).

By the end of laser pulse radiation by means of thermal expansion, the medium density reaches the value adequate to heating level, pressure in the sphere center reduces to virtually zero (curve 2, Fig. 3(b)). But, in virtue of process inertia and presence of overheated areas on granule periphery, the particles motion is continued in positive direction from center to periphery (curves 2 and 3, in Fig. 3(c)) that leads to formation of high pressure area on granule periphery (curve 2 in Fig. 3(b)).

Further, the particles outflow from central area leads to density decreasing and adequate pressure reduction, a level of which is significantly lower than initial one (curve 3 in Fig. 3(b)). This process underlines a formation of the negative half-period of longitudinal spherical acoustic wave.

Subsequently (as discharged area is formed in the center), the direction of particles motion is changed to reverse – from periphery to center (curve 4 in Fig. 3(c)). The process of bipolar acoustic wave formation is completed (curves 4 in Fig. 3(b) and (c)). In the following period, the acoustic wave is propagated at a sound speed (about 1500 m/s) to space that surrounds light-absorbing particle with consequent amplitude loss.

Hydraulic-mechanic effect of this acoustic wave on trabecula matrix (original hydraulic massage) forms the basis of SLT method of trabecula functions activation described above. Depending on indications and operation stages, the laser radiation capacity can be selected both at sub-threshold level (therapeutic action) and at level where local mechanical destruction of trabecula matrix is provided near to specific pigmented granules (threshold level).

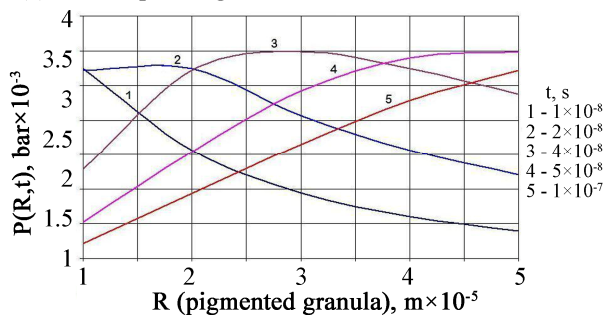
Numerical modeling of the parameters dependence of photoacoustic response of medium to laser effect on laser pulse duration was performed. Summarized calculation results of the main required dependences are given in Fig. 4(a) and 4(b). As in previous cases, the values of pressure amplitude P in negative phase of acoustic wave are normalized to granules irradiation

Table 1 Value of the acoustic relaxation time τ_{acr} for pigmented granulas model with different diameter [30].

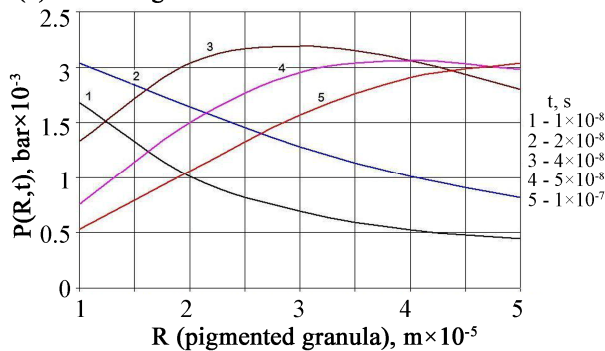
$2R, m$	$2 \cdot 10^{-5}$	$4 \cdot 10^{-5}$	$6 \cdot 10^{-5}$	$8 \cdot 10^{-5}$	$1 \cdot 10^{-4}$
τ_{acr}, s	$1.33 \cdot 10^{-8}$	$2.66 \cdot 10^{-8}$	$4.0 \cdot 10^{-8}$	$5.33 \cdot 10^{-8}$	$6.66 \cdot 10^{-8}$

$$E = 10^3 \text{ kW/m}^2.$$

(a) Central part of granula



(b) Board of granula



(c) External border of pigmented granules

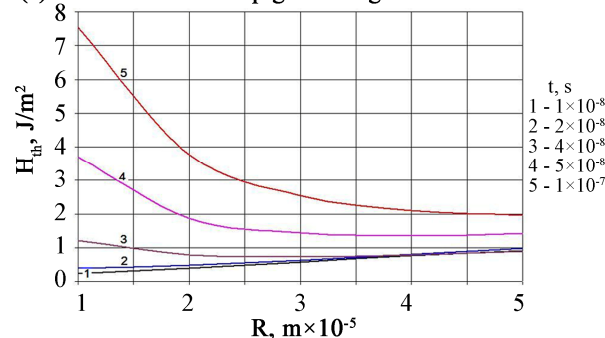


Fig. 4 (a) The theoretically simulated pressure amplitude dependence in the negative phase of acoustic wave on pigmented granula size (here – granule radius R) in the center of model sphere $P(r=0,t)$ during laser pulses radiation with different duration t (scale is on the right); (b) the theoretically simulated dependence of pressure amplitude at the border between pigmented granule and trabecula matrix $P(r=R,T)$ in the negative phase of acoustic wave on pigmented granula size (here – granule radius R) in the center of model sphere $P(r=0,t)$ during radiation by laser pulses with a different duration t (scale is on the right); (c) threshold radiant exposure of the cavitation (mechanical) destruction of

model structure is as a function of laser pulse duration t and radius of the pigmented granule of trabecula R .

Substantially, data of Fig. 4(a) related to the spherical model center of pigmented granule shows energy conversion efficiency of the laser radiation pulse into mechanical energy of the photo-stimulated acoustic wave.

This conversion can be when:

$$t < \tau_{acr}; \quad \tau_{acr} = \frac{2R}{c}, \quad (13)$$

where τ_{acr} is acoustic relaxation time of heated object and it is defined by quotient of characteristic dimension value of this object (in our case – $2R$) by sound speed in the medium – s (here $s \approx 1500 \text{ m/s}$). Values τ_{acr} for considered pigmented granulas sizes are given in Table 1.

Commenting on data of Fig. 4(a), we note that efficiency of the radiation energy conversion into mechanical energy of acoustic oscillation is defined (within the proposed model) by two factors.

First of all, it is fulfillment of condition (13). The second essential factor is a value dependence of the average value of volume density for absorbed light energy Q (5) on size (volume) of the light-absorbing particles. Physically, this is due to the fact that when values of the absorption spectral index correspond to pigmented granula properties, radiation is absorbed in the near surface layer and then light energy converted into heat is redistributed due to thermal conductivity at full volume of a particle. In accepted approximations this process is completed by the end of radiation pulse. Thus, Q value decreases with volume increasing and it causes adequate amplitude reduction of the generated acoustic wave. This factor influence is clearly showed by 1 and 2 curves in Fig. 4(a). Here, condition (13) is performed for the whole considered range of the calculation radiuses of light-absorbing particles and influence of pigmented granula volume is observed in relatively simple form that is showed in the Table 1.

The influence of non-compliance t with condition (13) requirement rises with increasing the laser pulse duration t (curves 3-5 in Fig. 4(a)). Physically, it indicates that for relatively long-time radiation of the pigmented granule, the formed acoustic wave is able to distribute beyond irradiated area. At that, light energy used to form acoustic wave is consumed irrationally and can lead to undesirable effect on surrounding areas. Adverse effect of this factor is especially evident during

radiation of the light-absorbing particles with a small diameter (curves 3-5 in Fig. 4(a)).

As the object of laser radiation is acoustic wave effect on optically transparent trabecula matrix, the study should take into account a natural amplitude loss of spherical acoustic wave during its distribution from model center to periphery of pigmented granule and further to trabecula matrix (Fig. 3(b), curves 2-4). The appropriate calculation of the acoustic wave amplitude in the negative phase at the border between pigmented granule and trabecula matrix are given to assess an impact of this factor. Results are shown in Fig. 4(b). Radiation conditions are similar in dependence calculation shown in Fig. 3(b).

Dependences in Fig. 4(a) and 4(b) show the presence of relatively narrow range of the laser radiation pulses duration (here it is about $3.5 \cdot 10^{-8}$ - $4.0 \cdot 10^{-8}$ s) that provide the most homogeneous and stable generation of photo-stimulated acoustic waves with characteristic dimensions from about 10 to 50 μm . Specified pulse duration corresponds to time of the particles acoustic relaxation τ_{acr} , whose characteristic dimensions are adequate to maximum of statistically significant normal (Gaussian) distribution (see Table 1 [30]).

Based on given model, the threshold conditions of the mechanical (cavitation) trabecula matrix photodamage were studied, as the most important radiation parameter when selecting mode of tissue radiation and stating technical requirements to appropriate laser medical equipment is radiant exposure realized in pulse H (J/m^2).

Selection criterion of this parameter in specified cases is a value defined as minimal density of radiation energy (exposure) that causes irreversible changes of biological tissues with probability of 0.5 for a certain period of time (normally 1 hour).

It is designated as HD_{50} (J/m^2). There are strict techniques of this value measuring while carrying out medical and biological studies [30]. In laser surgery practice, this value is defined approximately and as a rule measures an individual damage threshold of irradiated tissues of the specific object. It is designated as H_{th} (J/m^2) or W_{th} (J) (th - threshold). For objects of eye anatomy type containing high water concentration (90% and higher) while carrying out preliminary studies, distilled water or gels based on it are used as a model medium [32]. Particularly, as it shown in paper [32], the affecting by pulses with a duration of about 14 ns on the similar model medium with optical absorption coefficient $k(\lambda) = 3 \times 10^4 \text{ m}^{-1}$, cause medium photodamage that has not thermal nature (heating is about 12°C), but cavitational one. Cavitation (mechanical) damage in the model medium is realized in the negative phase of the photo-stimulated acoustic wave similar to shown in Fig. 3(b). Calculated pressure amplitude that corresponds to cavitation damage threshold of model medium was about minus 40 bars.

Subsequently, the optimal conditions of cavitation damage containing water of modal medium were defined [19]. Selection of special modes for radiation

allowed decreasing amplitude of negative pressure corresponding to destruction threshold about up to 10 bars. However, taking special measures to decrease cavitation threshold is devoid of sense for solving the problem of therapeutic action. The more relevant is selection of radiation modes which provide generation of the homogeneous acoustic wave by pigmented granules with a different diameter without loss of the conversion efficiency of radiation energy into energy of medium mechanical oscillations.

Due to given amplitudes of the negative half-wave of acoustic oscillations, an adequate amplitude for cavitation damage threshold containing water of modal medium P_{th} was accepted as equal to minus 40 bars according to this paper data [32]. Calculated dependences $H_{th}(R, t)$ which correspond to this condition at the external border of pigmented granules are given in Fig. 4(c).

According to data given in Fig. 4(b), the most homogeneous reaction of pigmented granules with a different diameter is realized when laser pulse duration is 30-40 ns. In this case, the high efficiency of radiant energy conversion into acoustic wave energy is kept.

Energy efficiency reduction and loss of homogeneous effect on granule with a different diameter are resulted from extension of granule weight influence and violation of condition defined by formula (13) as in the previous case.

4 Experimental study

4.1 IOP measurements

The results of IOP measurements have been presented on the (Fig. 5(a), No. 1). Eye (Fig. 5(a), No. 2 and 5(b)).

A board of the impression contact Schiotz tonometer that recorded changing of eye pressure as time passed was placed on cornea. In the process of measuring, there was sclera point irradiation by a laser. Each new measurement was performed at initial IOP that was equal to 5 Schiotz units or 31.5 mm of mercury. The mode with power of 0.9 W, pulse duration of 200 ms, pulse repetition rate of 2.5 Hz and radiation time of 4 seconds was selected for study of the laser effect on paralimbal zone of sclera depending on number of applications. Maximum temperature dynamics at selected laser effect is given in Fig. 1 (curve 2). Fig. 6 shows a nonlinear dependence of IOP dynamics depending on duty cycle of mode for modes with Fig. 2. It is apparent that mode with duty cycle of 50% that corresponds to curve 2 in Fig. 2 is the best. Standard deviation for experimental data is equal to 0.2 mm Hg.

Measurement results of sclera hydraulic conductivity at contact effect on it by pulse-periodic radiation ex-vivo showed that maximum increase of the eye sclera hydraulic conductivity of experimental animals in comparison with intact tissue was received at contact irradiation by fiber optics with a diameter of 600 μm , when power density was $1.8 \text{ W}/\text{cm}^2$, pulse duration $T=200$ ms, pulse repetition rate was 2.5 Hz and

radiation time was 4 seconds in selected ranges of the variation parameters of laser effect.

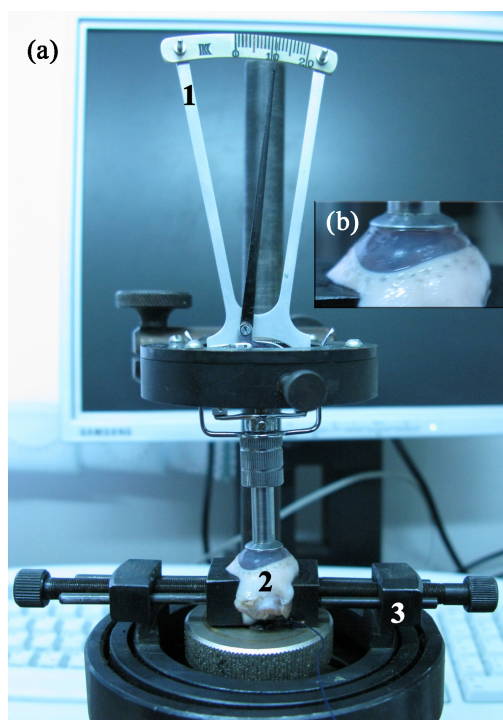


Fig. 5 (a) Experimental IOP measurement of selected mini-pig eye. No. 1 – impression contact Schiotz tonometer; No. 2 – mini-pig eye; No. 3 – special instrumental table suitable for IOP control, (b) zoomed mini-pig eye during IOP measurement with irradiated circle area (clearly visible dots).

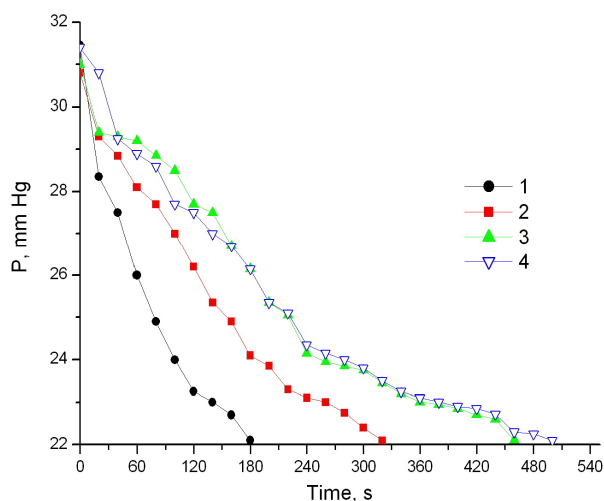


Fig. 6 Experimental IOP dynamics depending on duty cycle of the time mode for power of 0.9 W. Pulse duration is 200 ms, duty cycle is equal to 2 (curve 1), 1.3 (2), 2.6 (3), 4 (4); standard deviation is equal to 0.2 mm Hg.

4.2 Study of light propagation dynamics in the course of laser irradiation of sclera

Previously constructed and described in the paper [20] laboratory setup was modified to study changing of the interscleral structure due to measurement of light diffusion dynamics during laser effect ex-vivo. This setup included the fiber optics system that enabled to irradiate a sample perpendicular to its surface and to detect passed signal through fixed sample during irradiation by receiving device. With motion of receiving fiber optics perpendicular to sample irradiation direction on a back side, the radiation that passed through paralimbal zone and trabecular zone was both studied (Fig. 7).

Transmitted intensities had Gaussian distribution at that increasing of efficient Gaussian radius in passing through trabecular zone (Fig. 7, curve 2) was insignificant (by 15-20% in regard to decreasing radiation), when passing through paralimbal zone (Fig. 7, curve 1), increasing of efficient Gaussian radius varied from 4 to 6 times that was related to high heterogeneity and pigment presence. However, thickness of paralimbal zone for studied samples of mini-pigs sclera was 2 ± 0.5 times less.

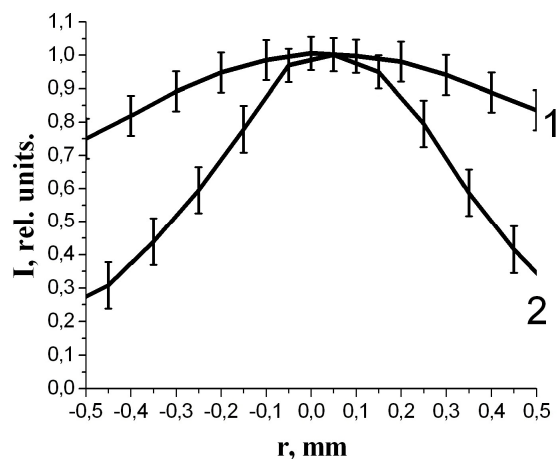


Fig. 7 Experimental intensity distribution of radiation passed through samples with standard deviation. 1 – paralimbalzone, 2 – trabecularzone.

4.3 Study of hydraulic conductivity around laser applications

Immediately after study of passed radiation intensities for the same samples, the study of sclera hydraulic conductivity for trabecular and paralimbal zones was carried out. Liquid trickled to pressurized capillary through a sample on the opposite side of this sample being in contact with liquid only by one of its side (to exclude seepage from lateral surfaces).

Irradiated and intact trabecular and paralimbal sclera zones were studied. Hydraulic conductivity was increased for both zones (Table 2).

Coefficient of sclera hydraulic conductivity (K) is calculated by formula:

$$K = (Q/S)/(\Delta P/h) = l * h / (\Delta P * t), \quad (15)$$

where Q is volume of bled liquid through a sample, S is capillary section; l is height of liquid column in capillary; h is sclera thickness in bleeding place; ΔP is differential pressure; t is bleeding time.

Table 2 Experimental coefficient of sclera hydraulic conductivity (K) for trabecular (TR) and paralimbal (PL) zones, obtained from experiments of hydraulic conductivity around laser applications.

Zone	K (m ⁴ /ns)
PL intact	3.4·10 ⁻¹⁴
PL irradi.	6.28·10 ⁻¹⁴
TR intact	0.8·10 ⁻¹⁴
TR irradi.	2.38·10 ⁻¹⁴

Measurement of bled liquid volume through capillary section S at regular intervals $\Delta t \sim 5$ min showed linear dependence of this volume on time that corresponded to Darcy law. Furthermore, the maximum extension of sclera thickness was observed in maximum value of the angular and radial component difference of stresses tensor received in model given in Section 3.3.

It was shown that when the distance between zones centers effecting on trabecular and paralimbal zones by fiber optics with a diameter of 600 μm was more than four radiuses of fiber optics (2400 μm), effect was non-additive, so there was no mutual exposure and enhancement of structural changes in both zones.

5 Conclusions

Study presented in this paper covers two existing in nature tracts of the intraocular fluid outflow from eye – uveoscleral and trabecular. Dependences of the hydroacoustic response parameters of trabecular medium on pigmented inclusions sizes and radiation time were calculated. For relatively long-time radiation of the pigmented granule, the formed acoustic wave is able to distribute beyond irradiated area. At that, light energy used to form acoustic wave is consumed irrationally and can lead to undesirable effect on surrounding areas. Adverse effect of this factor is especially evident during radiation of the light-absorbing particles with a small diameter.

Developed theoretical models of heating processes can be used for preliminary definition of the laser settings in ex-vivo experiments: laser radiation modes which were found theoretically led to enhancement of the hydraulic conductivity of drainage system.

Developed models and their experimental verification provide an opportunity to calculate thermal stresses, predict development of cavitation events at repetitively-pulsed laser effect on eye sclera in its different zones and can be used to optimize laser medical technologies for glaucoma treatment.

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Optical clearing of human *dura mater* by glucose solutions

Elina A. Genina^{1,2}, Alexey N. Bashkatov^{1,2}, Valery V. Tuchin^{1,3,4}

¹ Saratov National Research State University, 83 Astrakhanskaya Str., Saratov 410012, Russia

² National Research Tomsk State University, 36 Lenina Ave., Tomsk 634050, Russia

³ Laboratory of Laser Diagnostics of Technical and Living Systems, Institute of Precision Mechanics and Control, Russian Academy of Sciences, 24 Rabochaya Str., Saratov 410028, Russia

⁴ Samara National Research University, 34 Moskovskoe Shosse, Samara 443086, Russia

* e-mail: eagenina@yandex.ru

Abstract. Significant light scattering by *dura mater* in the visible spectral range seriously restricts the application of noninvasive optical methods to the diagnostics and therapy of brain diseases. We present the results of the study of human *dura mater* optical clearing *in vitro* by the glucose solutions with the concentrations 1.5M and 3M in the spectral range 400-700 nm. The efficiency of the tissue optical clearing is assessed. Basing on the analysis of the kinetics of collimated transmittance of light by samples of *dura mater*, the relative glucose diffusion coefficients are calculated that amount to $(1.1 \pm 0.1) \times 10^{-6}$ cm²/s and $(2.0 \pm 0.2) \times 10^{-6}$ cm²/s, respectively. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Optical immersion clearing; *Dura mater*; Collimated transmittance; Diffusion coefficient.

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1 Introduction

The human *dura mater* (DM) is a protective membrane surrounding the brain [1]. It is a typical fibrous tissue. The bundles of collagen fibrils in the stroma, the layer that forms the main part of the membrane, are arranged in a few rows, interlacing to form a mesh [2, 3]. The mean diameter of collagen fibrils assessed using the electron microscopy data, amounts to 100 ± 5 nm [4]. A network of elastic fibres surrounds the collagen layer. The collagen and the elastic bundles are surrounded by the amorphous interstitial matrix [2, 3] that consists of the components typical for this fibrous tissue, including proteins, proteoglycans, glycoproteins, *etc.* [5].

The thickness of DM varies from 0.3 to 0.9 mm depending on the age. The age-specific features of the DM structure are mainly reduced to the changed thickness of layers, interrelation and orientation of fibrous elements, cell density, and microcirculatory bed development [2, 3].

Since the collagen layer is the basic DM layer, its optical properties, i.e., sufficiently high scattering coefficient and relatively low absorption coefficient in the visible and near IR range, determine the optical properties of the entire DM [6]. The main chromophores of DM are haemoglobin and water.

Noninvasive diagnostics and sparing therapy of brain diseases are urgent problems of modern medicine. Optical methods can be rather efficient in solving these problems [7-9]; however, the significant scattering of visible light in DM seriously restricts their application in the diagnostics and therapy of brain diseases.

One of the simplest and most efficient ways to increase the probing depth and quality of images and to improve the accuracy of spectroscopic information from the blood circulation network and the structures of the cerebral cortex is to reduce the light scattering in DM for some time [10, 11]. The reduction of scattering would also decrease the divergence and provide better light beam focusing in cerebral surgery, which could essentially improve the efficiency of diagnostic

procedures and reduce the energy of radiation, necessary for providing the required light effect [12, 13].

The reduction of light scattering in a fibrous tissue is based on matching the refractive indices of tissue components either by partial replacement of the interstitial fluid with the immersion agent, having a higher refractive index, or by increasing the concentration of proteins and mucopolysaccharides in the interstitial fluid due to water diffusion from the tissue, caused by osmosis. In this case, the refractive index of the interstitial fluid approaches that of the scattering fibrils. Moreover, the optical homogeneity of the tissue can increase due to denser packing of collagen fibres under the conditions of dehydration caused by immersion agents [14].

At present, different hyperosmotic liquids are used as immersion agents, e.g., the solutions of glycerol, propylene glycol, some radiopaque substances (trazograph, verographine, Omnipack), *etc.* [14]. For their clinical application, one should consider both their biocompatibility and their diffusion rate in the tissues. One of the promising candidates for the role of immersion agent is the aqueous glucose solution, which is allowed for clinical use [15], possesses sufficiently high refractive index and, as shown in other fibrous tissues [16-18], diffuses into a tissue with sufficient rate.

The aim of the present paper is to study the optical clearing of human *dura mater* by aqueous solutions of glucose having different concentrations.

2 Materials and methods

The samples of human DM were obtained in 24 hours *post mortem* and were kept at the temperature -12°C . For the experimental study, the 10×15 mm samples were cut from the tissue. Eight samples of the tissue with the average thickness of 0.52 ± 0.09 mm were used to study the optical clearing of DM caused by immersion liquids. The thickness of the samples was measured using a micrometer; the measurement

accuracy was ± 0.01 mm. The samples were divided into two equal groups. The first group was used to measure the weight of the samples in the process of optical clearing. The second group was used to study the kinetics of the collimated transmittance spectra under the effect of immersion agents.

All procedures were performed in accordance with "Rules for Conducting Qualitative Clinical Trials in the Russian Federation" (approved by the Ministry of Health of the Russian Federation and enacted on January 1, 1999), appendix 3 to Order No. 755 of the Ministry of Health of the USSR of 10.08.77, the provisions of WMA Declaration of Helsinki (2000) and the recommendations contained in the European Community Directives (No. 86 / 609EC).

As optical immersion agents we used the aqueous solutions of glucose with the concentration 1.5M (0.27 g/ml) and 3M (0.54 g/ml), prepared from the powder glucose monohydrate (ChemMed, Russia). The refractive indices of the solutions, measured using the Abbe refractometer IRF-454B2M (LOMO, Russia) at the wavelength 589 nm, amounted to 1.367 and 1.398; the pH of the solutions, measured using the pH-meter Hanna (Germany), was equal to 6.0 and 4.7, respectively.

To measure the weight of DM samples, we used the electronic balance SA210 (Scientech, USA) with the accuracy of ± 0.001 g. The samples were placed in a cuvette with immersion agents and then every minute were taken out and weighed. The excess liquid was removed using the filtering paper. The weight measurements were repeated during 30 minutes.

The spectra of collimated transmittance of the tissue samples were measured using the multichannel spectrometer USB4000-Vis-NIR (Ocean Optics, USA). The tissue sample, fixed on the 3.5×1.5 cm² plastic plate with the 8×8 mm² hole in the centre, was merged in the glass cuvette having the volume 5 ml filled with glucose solution. The cuvette was installed between two optical fibres QP400-1-VIS-NIR (Ocean Optics, USA) with the inner diameter 400 μ m. To provide the beam collimation the collimators 74-ACR (Ocean Optics, USA) were fixed at the faces of the fibres using the standard connectors SMA-905. As a source of radiation, the halogen lamp HL-2000 (Ocean Optics, USA) was used. The collimated transmittance spectra were recorded every 20 s during 15-20 minutes, starting from the moment of placing the DM sample in the glucose solution. The measurement error did not exceed 5% of the measured quantity in the wavelength range above 500 nm and 10% at shorter wavelengths. Before and after the spectral measurements, the thickness and the weight of the samples were measured. All measurements were carried out at the temperature 20°C.

The relative coefficients of glucose diffusion were calculated basing on the analysis of kinetics of the collimated transmittance of the DM samples, placed in the glucose solution. In the considered case, the relative diffusion coefficient characterises the mean rate of the exchange flux of the clearing agent (glucose molecules)

into the tissue and the water flux from the tissue. The interstitial matrix of fibrous tissues can be presented as a polyelectrolyte gel [19-21]. Hence, the diffusion process can be described using the well-developed technique of physical and colloid chemistry [22]. According to this approach, the mobility of molecules in binary systems is characterised by the partial self-diffusion coefficients of the solvent molecules (in the present case, water) and the molecules of dissolved substance (glucose). The fluxes of molecules of the solvent and the dissolved substance interact with each other, and the corresponding diffusion coefficients are coupled via the interdiffusion coefficients [22]. Unfortunately, at present, there are no reliable methods for separate measurement of these coefficients, or they can be measured only in rare particular cases. In the present case of analysing the diffusion of high-concentration substances in tissues, one can consider only the relative diffusion coefficient.

The time dependence of DM swelling degree $H(t)$ was approximated by the phenomenological expression [23, 24]:

$$H(t) = \frac{M(t) - M(t=0)}{M(t=0)} = A_w (1 - \exp(-t/\tau_{sw})), \quad (1)$$

where $M(t)$ is the DM sample mass at different moments of time in the process of swelling, A_w and τ_{sw} are the phenomenological constants describing the process of swelling under the action of the glucose solution. Then the variation of the tissue volume can be expressed in the following form:

$$V(t) = V(t=0) + H(t)M(t=0)/\rho_{gl} = V(t=0) + \frac{M(t=0)}{\rho_{gl}} A_w (1 - \exp(-t/\tau_{sw})), \quad (2)$$

where $\rho_{gl} = 1.157$ g/ml is the glucose solution density [25]. Eq. (2) can be rewritten in the simpler form:

$$V(t) = V(t=0) + A(1 - \exp(-t/\tau_{sw})), \quad (3)$$

where $A = \frac{M(t=0)}{\rho_{gl}} A_w$. The volume of the DM sample changes mainly due to the variation of its thickness $l(t)$, which can be expressed as

$$l(t) = l(t=0) + A^* (1 - \exp(-t/\tau_{sw})), \quad (4)$$

where $A^* = A/S$, and S is the area of the sample in cm².

The sample thickness l was calculated at each moment of time using Eq. (4). The weight measurements of the DM samples were performed to estimate the constants A^* and τ_{sw} .

The process of glucose transport in DM was described within the frameworks of the free diffusion model. The following assumptions were made concerning the transport process: i) only the concentration diffusion takes place, i.e., the exchange flow of glucose into the tissue and the water from the tissue at a given point is proportional to the gradient of glucose concentration at this point; ii) the diffusion coefficient is constant at all points inside the studied tissue sample.

Geometrically the DM sample can be presented as a plane-parallel plate having the finite thickness. Since the area of the upper and lower surfaces of the plate considerably exceeded its side surface area, it is possible to neglect the edge effects and to solve the one-dimensional diffusion problem described by the equation

$$\frac{\partial C(x, t)}{\partial t} = D \frac{\partial^2 C(x, t)}{\partial x^2},$$

where $C(x, t)$ is the glucose concentration in the DM (g/ml); D is the diffusion coefficient (cm²/s); t is the time of diffusion process (s); x is the spatial depth coordinate of the tissue sample (cm). Since in the experiments the glucose solution volume (≈ 3000 mm³) considerably exceeded the DM sample volume (≈ 100 mm³), the corresponding boundary conditions had the form $C(0, t) = C(l, t) = C_0$, where C_0 is the glucose concentration in the solution; l is the tissue sample thickness (cm). The initial condition reflects the fact that there is no glucose in the sample volume before the incubation beginning, i.e., $C(x, 0) = 0$.

The solution of the diffusion equation yields the mean concentration of the glucose solution in the sample at any moment of time [24]:

$$C(t) = C_0 \left(1 - \frac{8}{\pi^2} \sum_{i=0}^{\infty} \frac{1}{(2i+1)^2} e^{-\frac{(2i+1)^2 \pi^2 D t}{l^2}} \right), \quad (5)$$

which, in turn, makes it possible to use the relation $n_{gl} = n_{H_2O} + 0.1515 \cdot C_{gl}$ [26] for aqueous solutions of glucose, where n_{gl} is the refractive index of the glucose solution, n_{H_2O} is the refractive index of water, and C_{gl} is the glucose concentration in the solution (g/ml). Then we can express the time dependence of the refractive index of the interstitial fluid as $n_i(t) = n_{i0} + 0.1515 C(t) / (1 - \varphi)$. Here $n_i(t)$ is the refractive index of the interstitial fluid gradually replaced with the glucose solution; n_{i0} is the refractive index of the interstitial fluid at the initial moment of time, and φ is the volume fraction of the scatterers in the tissue; for DM $\varphi = 0.3$ [23].

The variation of n_i reduces the DM scattering coefficient μ_s , which for a system of scatterers having

the shape of infinite cylinders is expressed as [23, 24, 27]:

$$\mu_s(t) = \frac{\varphi}{\pi a^2} \sigma_s(t) \frac{(1 - \varphi)^3}{1 + \varphi}, \quad (6)$$

where σ_s is the scattering cross section. In the Rayleigh-Gans approximation for a system of infinite cylinders [28, 29] it is given by the expression

$$\sigma_s(t) = \frac{\pi^2 a x(t)^3}{8} (m(t)^2 - 1)^2 \left(1 + \frac{2}{(m(t)^2 + 1)^2} \right), \quad (7)$$

where $x(t) = 2\pi a n_i(t) / \lambda$ is the diffraction parameter, $m(t) = n_c / n_i(t)$ is the relative refractive index of the scatterers, n_c is the refractive index of DM collagen fibres, and $a \approx 50$ nm [23] is the radius of the scatterers.

Apparently, the swelling of tissue samples will lead to the change of the volume fraction of scatterers, and, therefore, of the packing factor and the number of scatterers per unit volume (see Eq. (6)). With Eq. (3) taken into account, the variation kinetics of the volume fraction of scatterers can be described by the expression [23]:

$$\varphi(t) = \frac{\varphi(t=0) \times V(t=0)}{V(t=0) + A(1 - \exp(-t/\tau_{sw}))}. \quad (8)$$

The time dependence of the collimated transmission coefficient of the DM sampled placed in the glucose solution has the form:

$$T_c(t) \approx \exp(-(\mu_a + \mu_s(t))l(t)), \quad (9)$$

where μ_a is the DM sample absorption coefficient. In the calculations, we used the values of absorption coefficients obtained in Ref. [10].

Eqs. (4)-(9) determine the dependence of the collimated transmission coefficient on the concentration of the glucose solution in the sample of DM, i.e., formulate the direct problem. The inverse problem in this case is the reconstruction of the diffusion coefficient from the kinetics of collimated transmission. We solved this problem by minimising the target function

$$f(D) = \sum_{i=1}^{N_i} (T_c(D, t_i) - T_c^*(t_i))^2, \quad (10)$$

where N_i is the total number of experimental points, obtained in the course of recording the collimated transmittance kinetics at a fixed wavelength; $T_c(D, t)$ is the transmittance, calculated using Eq. (9) at the

moment of time t for the given value of D ; $T_c^*(t)$ is the experimentally measured value of the transmittance at the moment of time t .

To minimise the target function (10) we used the simplex method, described in detail in Ref. [30]. The iteration procedure was repeated until the matching of the experimental and calculated data. As a criterion of terminating the iteration process, we used the condition

$$\frac{1}{N_t} \sum_{i=1}^{N_t} \frac{|T_c(D, t_i) - T_c^*(t_i)|}{T_c^*(t_i)} \leq 0.01.$$

In more detail, the technique of calculating the relative diffusion coefficient is presented in Ref. [24].

3 Results and discussion

Figure 1 presents the time dependence of the weight of the samples, normalised to the initial value, under the effect of the studied glucose solutions. It is clearly seen that the glucose solutions significantly increase the weight of the samples by $40 \pm 7\%$ under the action of 1.5M solution and by $63 \pm 8\%$ under the action of 3M solution. The difference in swelling degree of the samples in Fig. 1 can be explained by the difference in pH of the used glucose solutions. The glucose solution having higher concentration causes the higher degree of tissue hydration.

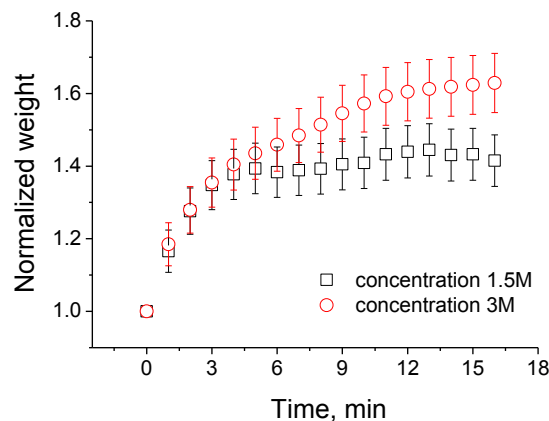


Fig. 1 Kinetics of the normalised weight of DM samples affected by the aqueous glucose solutions with the concentrations 1.5M and 3M.

As mentioned above, the DM stroma is a polyelectrolyte gel. If the tissue pH corresponds to the isoelectric point, then the attraction forces between the equal amounts of positive and negative charges (zwitter-ion pairs) [31] keep the tissue in the most dense state, and the degree of swelling in this case is minimal [19]. Earlier it was shown that the cornea collagen has the maximal degree of swelling at $\text{pH} = 4$, and the minimal one at $\text{pH} = 7$ [32]. Analogous results were obtained in the non-dried bovine cornea [33]. It is known that the average pH of the interstitial fluid amounts to 7.4 [20,

34, 35]. Thus, the fibrous tissues (in the present case DM) in their intact condition already possess a certain degree of hydration. With the shift of pH from the isoelectric point, the tissue hydration increases due to the decreased number of zwitter-ion pairs, and, correspondingly, the increased resulting static charge. Above the isoelectric point, the resulting charge is negative, and below this point, it is positive and can affect the tissue swelling in two ways. First, to support the electric neutrality in the stroma the static charge attracts a greater number of oppositely charged ions, which leads to the accumulation of small-scale ions in the interstitial space. This gives rise to the excess internal osmotic pressure and increases the swelling. Second, the decrease of the number of zwitter-ion pairs leads to the decrease of the attraction force and, thus, reduces the packing density of fibrils in the stroma, which also enhances the swelling [19].

Thus, the weight increase of DM samples can be caused by the replacement of water molecules with glucose molecules in the tissue due to, first, the greater molecular mass of glucose and, second, the additional hydration of the tissue because of decreased pH of the interstitial fluid.

Figure 2 presents the typical spectral and temporal dependences of the collimated transmittance of DM samples, affected by the studied glucose solution. The average values of the thickness and weight of the samples before and after the spectral measurements of collimated transmittance in the process of optical clearing are presented in Table 1.

At the beginning of the experiment, we observed the rapid growth of the collimated transmittance. The maximal transmittance was achieved nearly in 6 minutes after placing the sample in the cuvette with 3M solution and in 8 minutes for the 1.5M solution of glucose.

The figure shows that at the initial moment of time the DM samples were practically opaque for the optical radiation. In the process of replacing water molecules in the interstitial fluid with glucose molecules, we observed the increase of optical transmission, i.e., the DM transparency, which is due to the matching of refractive indices of the interstitial fluid and the collagen fibres. As a result, the scattering is reduced and the collimated transmittance increases.

From Fig. 2 it is seen that the process of interaction between the glucose solution and the DM tissue includes two stages. At the first stage, because of the partial replacement of water with glucose solution one can observe the gradual increase of the collimated transmittance in the DM samples. It is clearly seen that with the increased concentration of glucose in the immersion solutions the degree of the collimated transmittance increase also grows. This mainly results from the rise of the refractive index of the interstitial fluid with growing concentration of the solution and, therefore, the better matching of refractive indices between the interstitial fluid and the collagen fibres of the tissue.

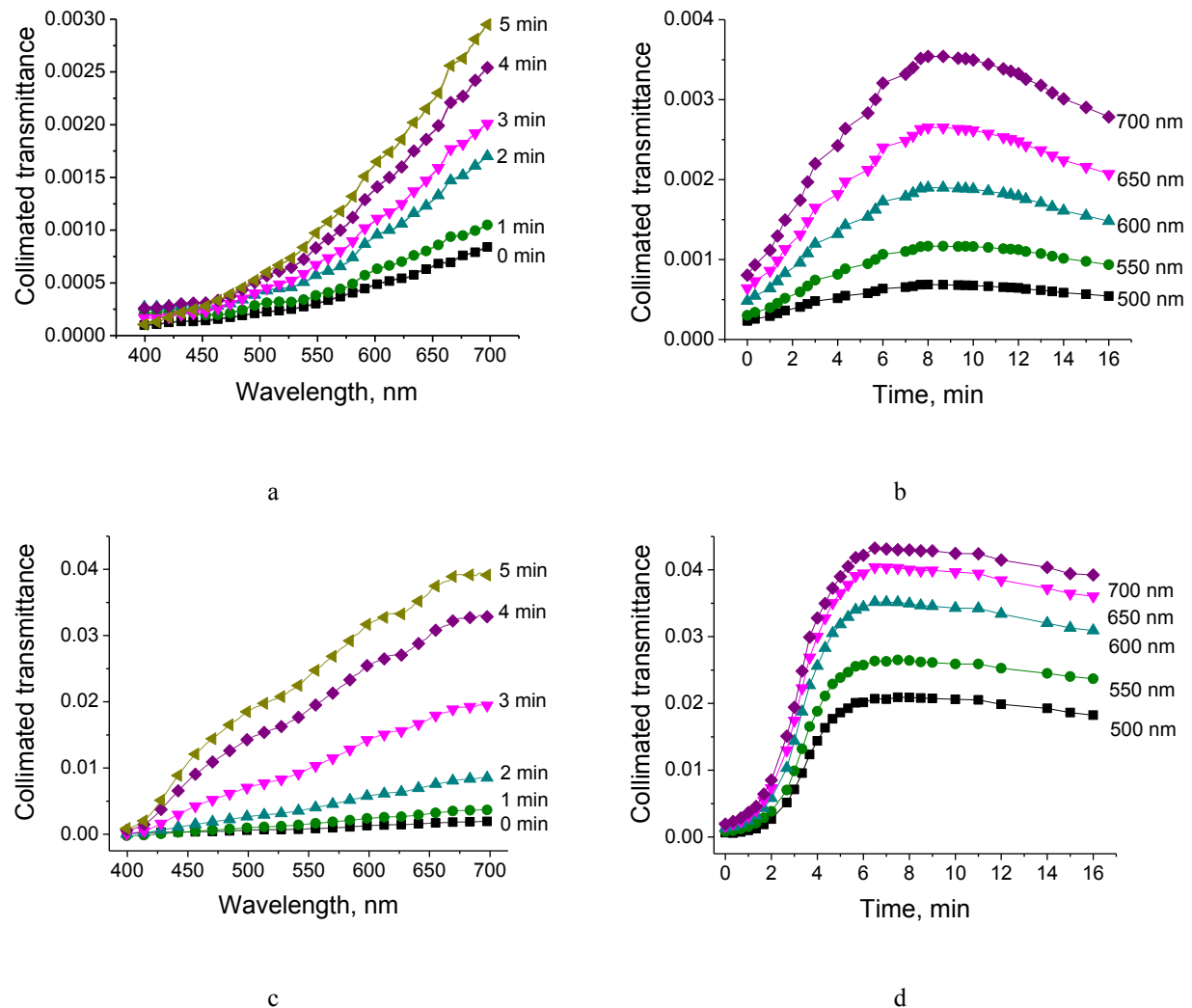


Fig. 2 Typical spectral (a, c) and temporal (b, d) dependences of the collimated transmittance of the DM samples under the action of glucose aqueous solutions with the concentrations 1.5M (a, b) and 3M (c, d).

Table 1 Thickness and weight of DM samples before (l_0 , w_0) and after (l , w) the spectral measurements of collimated transmittance in the process of optical clearing.

Concentration, M	pH	Thickness, mm		Weight, mg	
		l_0	l	w_0	w
1.5	6.0	0.56±0.02	0.59±0.01	97±5	115±3
3	4.7	0.46±0.01	0.58±0.02	101±3	120±4

As seen from Fig. 2, the second stage of interaction between the DM samples and the glucose solution is the reduction of collimated transmittance. To our opinion, the reason is the tissue swelling. From the data of Table 1, it follows that for pH close to the isoelectric point of proteins the thickness increase in the samples is insignificant. At lower pH, on the contrary, the tissue thickness increases stronger, which means that the swelling of the samples depends on the pH change in the interstitial fluid.

The tissue swelling affects the intensity of radiation transmitted through the tissue, which is caused by the increased sample thickness and, therefore, the decreased volume fraction of scatterers. As shown in Ref. [27], the dependence of the scattering coefficient μ_s on the volume fraction of scatterers (collagen and elastin fibrils) is described by Eq. (6). If, following Refs. [19, 20], we assume that the tissue volume increases mainly because of larger separation between the fibrils, while the fibril size does not change in the process of swelling during the observation time, then the parameters a and

σ_s can be treated as constants. Since we suppose that the process of immersion clearing is completed and all further variations of scattering kinetics are related only to the variations of the packing factor, the change of μ_s is determined by the behaviour of the function $\varphi(1-\varphi)^3/(1+\varphi)$, having a maximum at $\varphi=0.2$ (see Fig. 3). Since in fibrous tissues the value of φ is commonly about 0.3 [23], the decrease of φ leads to the increase of the scattering coefficient, which, in turn, leads to the reduction of the transmitted radiation intensity.

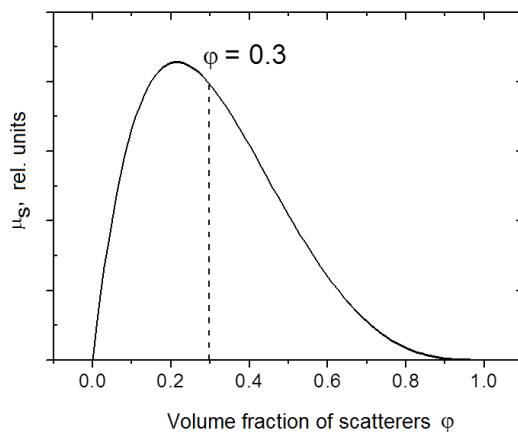


Fig. 3 The scattering coefficient versus the volume fraction of scatterers. The radius of collagen fibres and the scattering cross section are assumed constant.

The efficiency of optical clearing can serve as a parameter that allows the comparison of different optical clearing agents (OPA) with respect to the relative change of tissue transparency. Different definitions of the optical clearing efficiency are used depending on the measure parameters, e.g., the ratio of transport scattering coefficient values before the beginning of immersion and in some time after it [36], the ratio of the collimated transmittance values after the achievement of the maximal clearing and in the intact sample [37], the ratio of total transmission intensities, including the collimated component and the diffuse one, in some time after the beginning of clearing and at the initial moment [38], the maximal resolution of the object, visible through the tissue sample *in vitro*, and the relative variation of the parameter, characterising the signal-to-noise ratio by means of the contrast analysis of laser speckles *in vivo* [39].

From the data presented in Fig. 2 and Table 1, using Eq. (9) one can estimate the value of the attenuation coefficient $\mu_t = \mu_a + \mu_s$. Since the absorption coefficient μ_a in the visible spectral range is significantly smaller than the scattering coefficient μ_s , the change of μ_a in the process of optical clearing can be neglected. Then the degree (efficiency) of optical clearing can be evaluated using the expression

$$\Delta\mu_s = \frac{\mu_s(t=0) - \mu_s(t)}{\mu_s(t=0)} \times 100\% \quad (11)$$

where $\mu_s(t=0)$ is the original scattering coefficient for the intact tissue sample, $\mu_s(t)$ is the time-dependent scattering coefficient. To our opinion, the present method of evaluating the efficiency of optical clearing is rather objective, since it allows one to avoid the influence of the sample thickness on the registered transmitted signal [17].

The efficiency of DM optical clearing in different spectral ranges in 16 minutes after placing the samples in the studied glucose solutions is presented in Table 2. From the Table it follows that the optical clearing of DM samples occurred in the entire visible range of wavelengths. In Table 2, the values of the relative coefficient of glucose diffusion in DM are presented. The obtained values agree well with the results of estimating the glucose diffusion coefficient (0.2 g/ml) in DM [23] that amounts to $(1.63 \pm 0.29) \times 10^{-6} \text{ cm}^2/\text{s}$.

Different optical methods of measuring the glucose diffusion coefficients in biological tissues are widely presented in the literature. Since DM is a typical fibrous tissue, it is reasonable to compare the diffusion coefficients of glucose in DM and in other fibrous tissues, such as sclera and dermis. Thus, e.g., in Ref. [40] the values of the diffusion coefficient for the aqueous solutions of glucose with the concentrations 0.18, 0.30, and 0.40 g/ml in the human eye sclera *in vitro*, amounted to $(0.57 \pm 0.09) \times 10^{-6}$, $(1.47 \pm 0.36) \times 10^{-6}$ and $(1.52 \pm 0.05) \times 10^{-6} \text{ cm}^2/\text{s}$, respectively. In the sclera of rabbit eye *in vivo* the diffusion coefficient of the 40% glucose solution was $(0.54 \pm 0.1) \times 10^{-6} \text{ cm}^2/\text{s}$ [41]. In Ref. [17] the values of the diffusion coefficient for the aqueous solutions of glucose with the concentrations 30, 43 and 56% in the dermis of murine skin *in vitro* amounted to $(2.87 \pm 1.53) \times 10^{-6}$, $(2.70 \pm 2.22) \times 10^{-6}$ and $(1.40 \pm 0.96) \times 10^{-6} \text{ cm}^2/\text{s}$, respectively. Khalil et al. [42] have assessed the coefficient of glucose diffusion in human skin dermis as $(2.64 \pm 0.42) \times 10^{-6} \text{ cm}^2/\text{s}$. According to Ref. [43], the glucose (40%-glucose solution was used) diffusion coefficient in human skin *in vivo* amounted to $(2.56 \pm 0.13) \times 10^{-6} \text{ cm}^2/\text{s}$. Taking the difference of physical and optical properties of the studied tissues into account, we can conclude that the above values of the glucose diffusion coefficients agree well with the results, obtained in the present paper.

The effect of hyperosmotic OCA on the blood circulation in DM was studied in Ref. [44]. The authors of this paper have shown that the dehydrated glycerol reduces the blood flow velocity in the DM vessels by 20-30% compared to the normal blood flow and, therefore, glycerol cannot be used *in vivo*. The influence of glucose solutions on the blood flow is expressed much weaker and is reversible [45], which makes the glucose solutions more sparing to be used for optical clearing of DM.

Table 2 Optical clearing efficiency (Δn) in DM affected by glucose solutions in different spectral ranges, and the relative diffusion coefficient.

Concentration, M	Optical clearing efficiency, %			Diffusion coefficient, cm^2/s
	400-500 nm	500-600 nm	600-700 nm	
1.5	6.8±1.6	10.8±2.5	13.9±1.4	(1.1±0.1)×10 ⁻⁶
3	51.7±9.4	60.9±0.7	61.7±0.2	(2.0±0.2)×10 ⁻⁶

4 Conclusion

In the preset paper the process of optical clearing of *dura mater*, a typical fibrous tissue, is studied under the action of glucose solutions having the concentration of 1.5M and 3M. In the course of diffusion of glucose solutions into the interstitial space, a few processes having different rates are observed: the faster process of replacing the water molecules with glucose molecules, and the slower process of tissue swelling under the pH reduction in the interstitial fluid. The first process manifests itself in the rapid increase of the optical transparency of the tissue (6-8 min) due to the increase of the refractive index of the interstitial fluid and in the considerable increase of the sample weight. One can judge on the second process by the gradual decrease of the collimated transmittance of the samples after achieving the maximal degree of optical clearing and by

the increase of the sample thickness due to the swelling, which facilitates the increased scattering. In the paper, we calculated the efficiency of optical clearing of *dura mater* in three ranges of wavelengths under the effect of glucose solutions and estimated the relative diffusion coefficients of glucose.

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Comparison of a near-infrared reflectance spectroscopy system and skin conductance measurements for in vivo estimation of skin hydration: a clinical study

Inga Saknite^{1*}, Aleksejs Zavorins², Ilona Zablocka², Janis Kisis³, and Janis Spigulis¹

¹ Biophotonics Laboratory, Institute of Atomic Physics and Spectroscopy, University of Latvia, Raina Blvd. 19, Riga LV-1586, Latvia

² Faculty of Continuing Education, Riga Stradins University, Dzirciema Str. 16, Riga, LV-1007, Latvia

³ Department of Infectology and Dermatology, Riga Stradins University, Linezera Str. 3, Riga, LV-1006, Latvia

* e-mail: inga.saknite@lu.lv

Abstract. Diffuse reflectance spectroscopy system was developed for estimation of skin hydration in the near-infrared spectral range of 900-1700 nm. Experimental setup consisted of a near-infrared spectrometer, Y-type fiber optics probe with 1 detection and 6 illumination fibers, halogen-tungsten light source and a PC. By analyzing diffuse reflectance spectrum, a parameter representing skin hydration by performing baseline correction and calculating the area under the 1450 nm water absorption maximum is proposed. A clinical study was performed acquiring data of skin hydration of 39 patients' forearm skin. Results of the developed system are compared to results obtained by a commercial device based on skin conductance measurements. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: Diffuse reflectance; near-infrared; spectroscopy; water; absorption; hydration; skin.

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1 Introduction

1.1 Near-infrared reflectance spectroscopy

Near-infrared diffuse reflectance spectroscopy is a technique that has a potential to distinguish between different water volume fractions in skin as water has distinct absorption peaks in the near-infrared (NIR) and short-wave infrared (SWIR) spectral ranges. Thus, NIR and SWIR spectroscopy is useful and informative for distinguishing between different hydration levels in skin which is very interesting for the cosmetic industry, as well as for dermatologists. There are three main areas of research regarding diffuse reflectance spectroscopy in the NIR and SWIR spectral ranges: 1) integrating sphere based measurements for estimating *ex vivo* tissue absorption μ_a and scattering μ_s coefficients; 2) fiber probe based "point" measurements to acquire reflectance measurements collected over small localized tissue volumes both *in vivo* and *ex vivo*; 3) imaging measurements both *in vivo* [1-2] and *ex vivo* that offer

non-contact measurements of a larger area of tissues [3]. Previous studies based on fiber probe reflectance spectroscopy method have shown NIR and SWIR spectroscopy applications for distinguishing between *ex vivo* vulnerable and stable atherosclerotic plaques by estimating lipid-to-protein ratios [4-5], as well as distinguishing between *ex vivo* normal and cancerous breast tissue by estimating water and lipid volume fractions [6].

In our previous work [7] we presented a method to estimate skin hydration changes over a short period after application of different moisturizing creams based on near-infrared spectroscopy in the spectral range of 900–1700 nm. We proposed a skin hydration parameter, calculated as difference in absorbance between local water absorption maximum (1450 nm) and local water absorption minimum (1300 nm). It was possible to see hydration parameter changes over time after a moisturizing cream was applied on skin surface, however, we concluded that the sensitivity of the method should be improved.

1.2 Skin hydration

Skin is the largest organ of human body that has many important functions such as prevention of water loss. Skin consists of two main structural layers: epidermis and dermis. Epidermis consists of 5 sublayers, the outer of which, *stratum corneum* (SC), is the main barrier that protects the body from dehydration, as well as from other molecules (e.g. irritants) entering the skin [8].

Dry skin is characterized by redness (erythema), scaling and, in extreme cases, even fissuring. Individuals with dry skin often complain about a burning sensation or itch. The water content at the outermost layer of SC is normally 15-20%, in dehydrated or xerotic skin, on the other hand, it is only 10% [9]. Water content in SC is not homogeneous – it increases from around 15-30% at the outermost layer of SC to around 70% in the deeper living layer, and it stays at around 70% deeper in epidermis and dermis [10].

SC consists of dead keratinocytes also known as korneocytes (differentiated epidermal cells) that no longer possess a nucleus and are filled with keratin. Korneocytes also contain humectants or natural moisturizing factors (NMFs). These are molecules such as free amino acids and urea that are produced from a peptide called filaggrin. NMFs attract water molecules from dermal blood vessels to SC. This causes swelling of korneocytes, thus decreasing the inter-cellular space which is normally filled with hydrophobic lipids, e.g. ceramides, free fatty acids and cholesterol. This structure allows skin to keep the water inside the body, evading systemic dehydration. In addition, it does not allow toxic substances to penetrate the skin barrier. Transepidermal water loss (TEWL) is traditionally used to estimate the barrier function of the skin [11-12].

Application of moisturizing skin cosmetics reduces TEWL, maintains the physiological acidic pH level of skin surface and prevents degradation of intercellular connections and absorption of toxic substances. Skin moisturizers consist of three types of active substances: occlusives (petrolatum), humectants (urea, glycerin) and emollients (ceramides). Humectants attract water to SC from dermis; however, without the occlusive substances they could even increase TEWL. Occlusive moisturizers create a hydrophobic layer on skin surface that prevents the water that is attracted by humectants from leaving the corneal layer. Emollients are said to fill up the intracellular space in SC, thus reducing TEWL while also being less greasy than occlusive moisturizers [9].

2 Materials and Methods

In this study, we present a new experimental setup, as well as a new, more sensitive skin hydration parameter, compared to our previous work [7]. In addition, we did a clinical study in collaboration with Latvian cosmetics company MADARA. During this clinical study, we not only tested our developed experimental system based on NIR spectroscopy, but also compared results acquired by our system to the results acquired by a commercial device *DermaLab* (*Cortex Technology*, Denmark) that

estimates skin hydration based on skin conductance measurements. In this study, we used *DermaLab* with a flat faced probe where the electrodes organized as concentric rings. The measurement range of *DermaLab* is 0–999 μ Siemens, 1 μ Siemens resolution, 5% accuracy. During the clinical study, we tested the short-term (30 minutes) and long-term (1 month) effects of a new moisturizing cream developed by MADARA. The main components of the moisturizing cream are: Aqua/Water, Hippophae Rhamnoides (Sea-buckthorn) Fruit Extract, Sodium PCA, Urtica Dioica (Nettle) Leaf Extract, Linum Usitatissimum (Flax) Seed Extract, Paeonia Lactiflora (Peonia) Root Extract, Sodium Hyaluronate, and Hydrolyzed Hyaluronic Acid.

2.1 Experimental setup

Experimental setup consisted of a near-infrared spectrometer (NirQuest 512 by *Ocean Optics*) in the spectral range of 900-1700 nm, Y-type water-free optical fiber probe, and a stabilized tungsten-halogen light source (SLS201/M by *Thorlabs*) with radiation spectrum between 300 and 2600 nm (Fig. 1). This is a low-power light source (fiber-coupled optical power of 10 mW) corresponding to approximately 1 J/cm² which is considered a low dose [13]. As one measurement usually took approximately 1 second at a time, we believe, it didn't significantly increase the temperature of skin surface or cause any other change in skin physiology. Optical fiber probe consisted of 6 illumination fibers (each 400 μ m in diameter) in a circle around 1 detection fiber (400 μ m in diameter) without any separation. Due to very small separation between detection and illumination fibers (0,4 mm between the fiber centers), we expected to detect diffuse reflected signal from mainly the superficial layers of skin (the upper sublayers of epidermis).

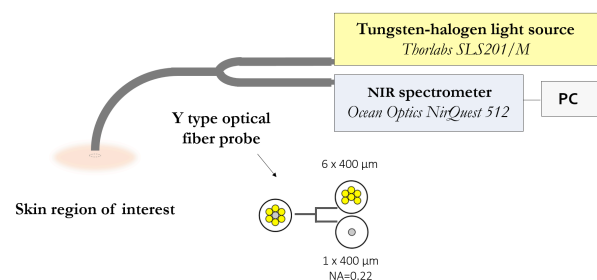


Fig. 1 Experimental setup for near-infrared diffuse reflectance measurements of skin.

Compared to the experimental setup we used in our previous study [7], in this setup the fiber probe end touched the surface of skin during the measurement. It is known that the compression of skin changes its optical properties [14-18]. As we were not able to include a pressure sensor in our experimental setup to determine the applied pressure for each measurement, the operator doing the measurements trained to apply approximately the same pressure for each measurement by just lightly touching the skin surface. In this way, we

believe, the applied pressure to skin surface was very low and the variability of all measurements was minimized.

2.2 Data analysis

We measured reflected intensity from skin surface I in the whole 900-1700 nm range by using the near-infrared spectrometer. We used a white reference tile *Avantes WS-2* that reflects 98% of the radiation in the spectral range 350-1800 nm to measure illumination spectrum I_0 ; in this measurement, the fiber probe end was kept at a 5 mm distance from the reference tile. We then converted reflected intensity values to optical density (OD) values based on Beer-Lambert-Bouguer law. OD values represent absorption in tissue due to mainly water and lipids in this spectral range. Correction for dark current (I_{dark}) was also applied:

$$OD(\lambda) = \log_{10} \frac{I_0(\lambda) - I_{dark}(\lambda)}{I(\lambda) - I_{dark}(\lambda)}. \quad (1)$$

A typical OD spectrum of skin in the spectral range of 900-1700 nm is shown in Fig. 2. There are three main absorption maxima: at around 980 nm, 1200 nm and 1450 nm. The first two (980 nm and 1200 nm) are from a vibrational overtone of the O-H bond [19], while the maximum at around 1450 nm is due to the first overtone of the O-H stretching [20].

For estimation of skin hydration, we chose the water absorption maximum at 1450 nm as it has the highest water absorption in this range, thus it is more sensitive to small changes in water volume fraction, compared to the other maxima. Due to high absorption around the water absorption maximum at 1450 nm, radiation around these wavelengths should not penetrate as deep in the tissue as other wavelengths in this spectral range, and in this study, we were mainly interested in the very upper sublayers of epidermis. We estimate that the radiation penetration depth around the water absorption maximum at 1450 nm is approximately 1 mm [21]. However, the measurement depth should be lower due to very small illumination and detection fiber separation (Fig. 1). Measurement depth of *DermaLab*, however, is much smaller – around 15 μm [22].

To eliminate scattering effect on OD spectrum, we used linear baseline correction [23] between local minima at 1280 nm and 1650 nm (Fig. 2). After baseline correction, we calculated the volume area under the 1450 nm maximum which we chose as a parameter representing skin hydration.

2.3 Clinical study

We performed a clinical study to compare our developed system based on NIR spectroscopy and a commercial device *DermaLab*. In this clinical study, we tested the short-term (30 minutes) and long-term (1 month) effect of a moisturizing cream (developed by MADARA Cosmetics, Riga, Latvia) on skin hydration. In total, 39 volunteers (females, age range 18 – 60,

average: 45, standard deviation: 12) participated in this study. The study protocol was approved by the local ethics committee, and each participant gave a written informed consent before the trial. A primary screening procedure was done by a dermatologist before the study. Measurements were performed in January and February in Riga, Latvia with outside temperature around 15 °C (January) and around 0 °C (February). During the measurements, room temperature was kept constant at around 23 °C, and all participants were asked to sit at rest for 15 minutes at room temperature before the start of the measurements. All measurements were performed by two different devices - the developed device for estimation of skin moisture level by near-infrared spectroscopy, and a commercial device *DermaLab* which estimates skin hydration based on changes in skin conductance (with an increased water volume fraction in skin, there is an increase in electrical conductance of skin).

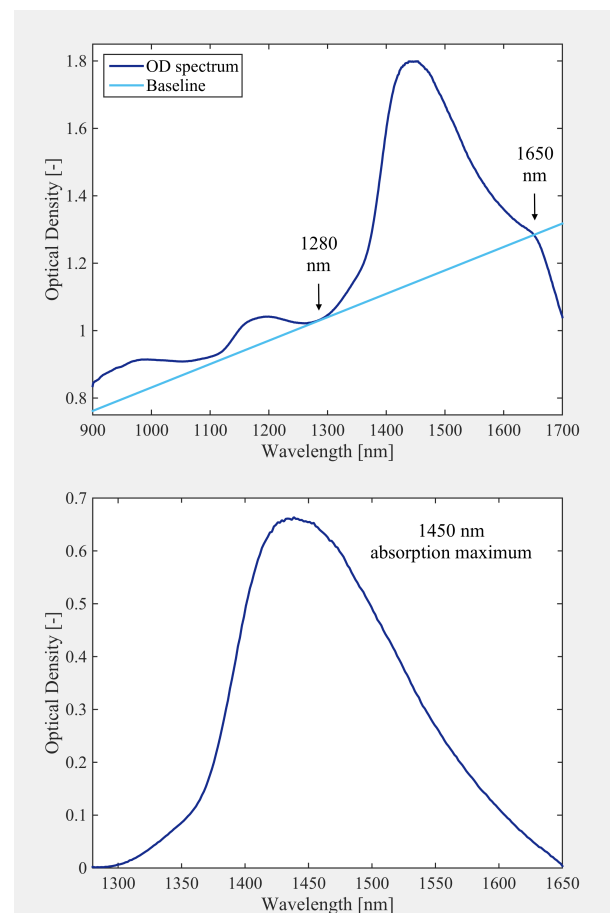


Fig. 2 Top: Example of an Optical Density (OD) spectrum of *in vivo* forearm skin and a baseline; Bottom: Baseline-corrected 1450 nm absorption maximum.

In the beginning, after the participant had sat still for at least 15 minutes, one measurement on right forearm (control) and three measurements on left forearm (Fig. 3) were performed by both devices. For the short-term (30 minutes) study, dermatologist applied the

Table 1 Results of the change in skin hydration parameter of the short-term and long-term clinical study - comparison of both devices (NIR device and *DermaLab*).

		NIR device	Two-sample T Test		<i>DermaLab</i>	Two-sample T Test
Short-term study	Control	ΔV	NO statistically significant difference	ΔV	-5%	Statistically significant difference
		SE		SE	1%	
	Cream	ΔV	Statistically significant difference	ΔV	+7%	Statistically significant difference
		SE		SE	1%	
Long-term study	Control	ΔV	Statistically significant difference	ΔV	+32%	Statistically significant difference
		SE		SE	5%	
	Cream	ΔV	Statistically significant difference	ΔV	+78%	Statistically significant difference
		SE		SE	13%	

moisturizing cream on the proximal part of volar aspect of left arm. Afterwards, the participant was asked to wait for 30 minutes in a room at a temperature of 23 °C, and another set of measurements were performed 30 minutes later - one measurement on right arm (control) and three measurements on left arm (where the cream had been applied). Three measurement points for left forearm were chosen due to possibly uneven application of the cream throughout the area. An average of the three measurements of left arm was later calculated and used for further analysis.

For the long-term study (1 month), participants were using the same moisturizing cream once a day on the same area of left forearm every day for one month. On the contrary, they were asked not to use any cream on the volar aspect of the right arm for the whole month. After 1 month, they came for another visit. After they had sat down still for at least 15 minutes to adapt to the room temperature, the same set of measurements was performed as in the beginning - one measurement on right arm (control) and three measurements on left arm where they had been applying the cream for the whole month.

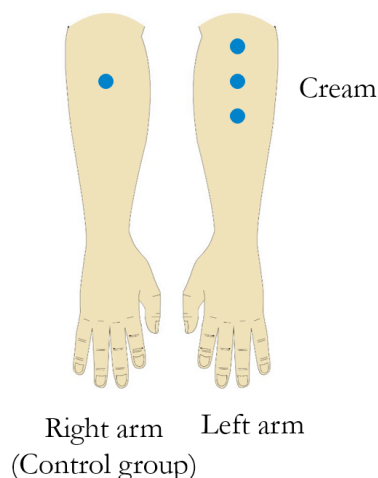


Fig. 3 Measurement points on the proximal part of volar aspect of right forearm (control group) and left forearm (cream group).

3 Results and Discussion

In results, separately for both devices, we calculated the increase (in percent) in skin hydration parameter 30 minutes/1 month later compared to the beginning of the study as the average for all 39 participants (ΔV), as well as the standard error (SE). At first, we performed Lilliefors test in *Matlab* to test whether each measurement group (e.g. data of control group in the beginning of the study of all 39 participants) corresponds to normal distribution. Afterwards, a Two-Sample T-Test was performed on two respective groups (e.g. control group in the beginning and control group 30 minutes later) to test whether there is a statistically significant difference between both groups, in other words, whether there is a statistically significant difference between results 30 minutes (or 1 month) later for all participants, compared to the beginning of the study. Results acquired by both devices are shown in Table 1.

ΔV is the increase (in percent) in skin hydration parameter 30 minutes/1 month later compared to the beginning of the study as the average for all 39 participants, SE is standard error.

In this study, we used a near-infrared spectroscopy system for estimation of skin hydration in the spectral range of 900-1700 nm during a clinical study involving 39 female participants. We compared results acquired by our developed NIR device to results acquired by a commercially available device *DermaLab* that estimates skin hydration based on skin conductance measurements. We also tested the short-term (30 minutes) and long-term (1 month) effect of a moisturizing cream developed by MADARA Cosmetics on forearm skin.

Results of the short-term study are quite different when comparing both devices. In Table 1, we can see that there is no statistically significant difference between control group in the beginning and 30 minutes later, measuring by NIR device, which is what we expected. However, there is a statistically significant difference between control groups before and after, measuring by *DermaLab* device; there is even a 5%

decrease in skin hydration parameter. On the other hand, results of both devices show a statistically significant increase in skin hydration parameter 30 minutes after application of the moisturizing cream: +4,5% increase by NIR device and +7% increase by *DermaLab* device. Even though the increase in percent is different for both devices, it is still comparable and shows the same trend for both devices.

Unexpectedly, results of the long-term study show statistically significant differences between both the control and the cream group by both devices. We expected an increase in skin hydration only for the cream group. These results suggest that there are other factors affecting skin hydration measurements, more so than the use of a moisturizing cream. Some of these factors might include the outside temperature (in the first set of measurements the outside temperature was around 15 degrees Celsius colder than in the second set of measurements one month later), use of different soap and/or other products on both forearms, physiological changes in skin etc. In future studies, when doing a long-term clinical study, measurements should preferably be done in similar external conditions such as room temperature and relative humidity.

We also tried to find a correlation between overall results by both devices. When comparing all patient data at the same temporal moment by both devices, we found very little correlation. Even though the trend is similar, at least for the short-term study (there is an increase in skin hydration 30 minutes after the cream was applied on skin surface), the results in general differ quite a lot between both devices. On one hand, it can be expected as both devices have different working principles and different measurement depths – while *DermaLab* measures skin hydration only in the SC (its measurement depth is around 15 μm), the measurement depth of NIR device is deeper and includes deeper sublayers of epidermis as well (possibly even upper dermis). After application of a moisturizing cream, water content is increased mainly in the SC (upper 10 – 20 μm in forearm skin), while it should not increase in deeper layers of epidermis. Because NIR device measures signal acquired from deeper epidermis, the increase of water content in the SC is less pronounced in the acquired overall signal. Therefore, *DermaLab* is expected to be more sensitive to even small changes in water content in SC.

The NIR device was developed in such a way to decrease the measurement depth as much as possible by arranging illumination and detection fibers without any separation between them. For analysis, only water absorption maximum of 1450 nm was used as it highly absorbs water, thus decreasing the penetration depth.

For future studies, cross polarizers might be incorporated in the experimental setup to reduce specular reflectance off the skin surface. Also, it might be useful to use illumination and detection fibers of a lower diameter (in this study, fibers of 400 μm in diameter were used), thus possibly reducing the penetration depth even more.

Unfortunately, there is no "golden standard" method to estimate skin hydration and compare results of our developed device to. Even though there are different commercially available devices for estimation of skin hydration based on electrical properties of skin (conductance, capacitance), we believe that optical methods might be useful and maybe even better for this purpose as they can work in a non-contact mode (e.g. NIR imaging) providing less effect of skin surface nonuniformity and roughness, dependence on contact pressure on skin surface, compared to devices based on electrical properties of skin. During the measurements by *DermaLab* device, we noticed that the results vary quite a lot when doing a measurement at approximately the same spot of forearm skin within a short period (less than half a minute). In some test measurements, the standard deviation between 5 measurements on the same spot was close to 20%. For NIR device, it was normally less than 2%. We also noticed another issue regarding measurements by *DermaLab* device – for a couple of participants, skin hydration measurement 1 month later showed an increase of up to 10 times, compared to the beginning of the study, which seemed a very unlikely result. We considered these results as faulty and did not include them in the calculation of the average value. The reason might be the use of some specific chemical substances on skin that systems based on conductance measurements are very sensitive to, even though none of the participants could recall being exposed to anything unusual.

Overall, we conclude that *DermaLab* is more sensitive to small changes in skin hydration, compared to our developed NIR device. One of the reasons might be the deeper measurement depth by NIR spectroscopy device. There is currently no standardized method for NIR spectroscopy application for estimation of skin hydration, thus more research in this field is needed.

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Finite difference methods for solving the transport equation in the problems of optical biomedical diagnostics

Leonid P. Bass^{1*}, Olga V. Nikolaeva¹, Alexander V. Bykov², and Mikhail Yu. Kirillin³

¹ M.V. Keldysh Institute of Applied Mathematics RAS, Miusskaya pl., 4, Moscow 125047, Russia

² Opto-Electronics and Measurement Techniques Lab., University of Oulu, P.O. Box 4500, Oulu 90570, Finland

³ Institute of Applied Physics RAS, Ulyanov St., 46, Nizhny Novgorod 603950, Russia

* e-mail: bass@kiam.ru

Abstract. The present paper is a retrospective review of the development of the approach to numerical modeling of radiation propagation in biological tissues, based on the use of finite difference methods for the solution of the radiative transfer equation (RTE) and its application to the problems of biomedical diagnostics. The advantage of finite difference methods is the possibility to obtain the solution of the direct problem of radiation propagation in turbid media, when the exact analytical solution is impossible. In turn, the possibility to solve the RTE opens wide perspectives for the solution of inverse problem and reconstruction of structural and functional characteristics of objects from the detected scattered radiation. We present a review of the finite difference methods applied to the solution of angiography problems and investigation of blood using optical biomedical diagnostic technologies. © 2017 Journal of Biomedical Photonics & Engineering.

Keywords: radiative transfer equation; finite difference techniques; Monte Carlo simulations; stationary problem; non-stationary problem.

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1 Introduction

The progress of modern optical biomedical diagnostics requires the development of models of probing radiation propagation in a biological object. In the radiative transfer theory, they distinguish between the direct problem, i.e., the calculation of the scattered intensity distribution basing on the object geometry and optical properties, and the inverse problem, i.e., the reconstruction of the optical and geometrical characteristics of the object from the measured scattered radiation. The solution of the inverse problem is the base of optical biomedical diagnostics.

A specific feature of biological objects is their complex geometry, characterised by different optical inhomogeneities. The basic equation of the radiative transfer theory (RTE) generally has no analytical solution for arbitrary geometry. Since biotissues are mostly strong scattering media for the radiation of optical range, the diffusion approximation is commonly used for the RTE, describing the propagation of radiation [1]. In the diffusion approximation, the RTE has analytical solutions for directional and isotropic sources of light and for infinite or semi-infinite homogeneous media. However, the use of diffusion approximation suffers from a number of restrictions; in

particular, it is hardly applicable at the distances from the source smaller than the transport length. For the arbitrary geometry the RTE in the diffusion approximation has also no analytical solution, however, the finite difference technique can be efficiently used for solving it [2]. Such approach has found wide applications in optical diffusion tomography, in particular, for the noninvasive diagnostics of breast cancer and functional diagnostics of human brain [2, 3]. An alternative approach is the numerical simulation using the stochastic Monte Carlo method [4]. This method is based on multiple calculation of random photon trajectories in the medium having the specified geometry and optical characteristics, followed by the statistical processing of the obtained results at the detection points [5, 6]. In particular, the Monte Carlo method was used to simulate the propagation of probing radiation in human head [6, 7] and laboratory animal [8]. The geometry obtained by MRT diagnostics can be used in this case as the initial data. However, both of the above approaches have their drawbacks. The numerical solution of RTE in the diffusion approximation does not provide sufficient accuracy near the source, and the Monte Carlo simulation is often accompanied by the statistical noise, the elimination of which requires high

computational expenses, which is not always acceptable, e.g., in solving inverse problems. In such cases, it is efficient to use finite-difference technique for solving RTE in its original form rather than in the diffusion approximation. The present paper presents a retrospective review of the approaches to the RTE solution by means of finite difference methods, including the cases of complex geometry [9-17]. The validation of approaches was based on the comparison of the results with those of Monte Carlo simulation. The developed approaches were used to solve the problems of angiography and optical diagnostics of blood. The finite difference methods can be also efficiently used to solve the direct problem in optical tomography [18].

2 Finite difference methods for solving the radiative transfer equation

2.1 Problem statement

The propagation of radiation from a pulsed source in a turbid medium is described by the linear integro-differential transfer equation [12]

$$\frac{1}{v} \frac{\partial \Psi}{\partial t} + \hat{L} \Psi(\vec{r}, \vec{\Omega}, t) = \hat{S} \Psi(\vec{r}, \vec{\Omega}, t) + Q(\vec{r}, \vec{\Omega}, t), \quad \vec{r} \in G, \quad (1.1)$$

where

$$\hat{L} \Psi(\vec{r}, \vec{\Omega}, t) = \vec{\Omega} \cdot \text{grad} \Psi(\vec{r}, \vec{\Omega}, t) + \Sigma_t(\vec{r}) \Psi(\vec{r}, \vec{\Omega}, t),$$

$$\hat{S} \Psi(\vec{r}, \vec{\Omega}, t) = \Sigma_s(\vec{r}) \int_{\Omega} \rho(\vec{r}, \vec{\Omega} \cdot \vec{\Omega}') \Psi(\vec{r}, \vec{\Omega}', t) d\vec{\Omega}'.$$

Here the function $\Psi(\vec{r}, \vec{\Omega}, t)$ describes the radiation flux density at the point $\vec{r}$ of the spatial domain G along the direction $\vec{\Omega} \in \Omega$. The quantity v is the velocity of photons in the medium. The extinction coefficient $\Sigma_t(\vec{r})$ determines the probability of interaction between the photon and the medium; the scattering coefficient $\Sigma_s(\vec{r})$ specifies the probability of changing the photon direction as a result of collision with atoms of the medium; the scattering indicatrix $\rho(\vec{r}, \vec{\Omega} \cdot \vec{\Omega}')$ describes the probability of changing the direction of photon propagation from $\vec{\Omega}'$ to $\vec{\Omega}$. At each spatial point this function is determined by the scalar product $\vec{\Omega} \cdot \vec{\Omega}'$, i.e., the cosine of photon deflection angle at each scattering event.

The function $Q(\vec{r}, \vec{\Omega}, t)$ determines the density of the source of photons that emits into a spatially limited cone at the initial moment of time (see Fig. 1). This function $Q(\vec{r}, \vec{\Omega}, t)$ has the form

$$Q(\vec{r}, \vec{\Omega}, t) = \frac{S_0}{2\pi(1 - \cos \alpha)} \cdot \delta(\vec{r} - \vec{r}_0) \times \times \eta \left(\frac{\vec{r} - \vec{r}_0}{|\vec{r} - \vec{r}_0|} \cdot \frac{\vec{r}_{ax} - \vec{r}_0}{|\vec{r}_{ax} - \vec{r}_0|} - \cos \alpha \right) \cdot \delta(t - t_0). \quad (1.2)$$

Here S_0 is the source power,

$$S_0 = \int_0^\infty dt \int d\vec{\Omega} \int d\vec{r} Q(\vec{r}, \vec{\Omega}, t);$$

δ is the Dirac delta function, providing the photon emission from the point $\vec{r}_0$ at the time moment t_0 ; the function $\eta(\alpha)$ specifies the radiation cone

$$\eta(a) = 0 \text{ for } a < 0, \quad \eta(a) = 1 \text{ for } a \geq 0;$$

α is the opening angle of the source, measured from the cone axis defined by the vector $\vec{r}_{ax}$, see Fig. 1.

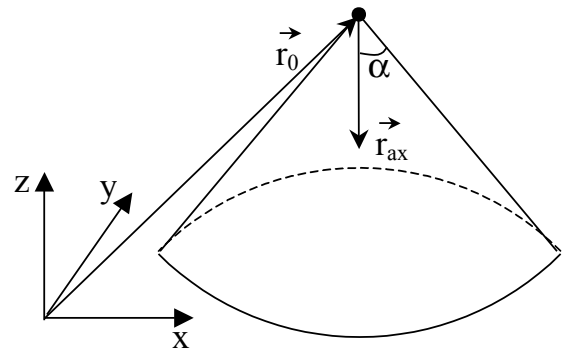


Fig. 1 Geometry of the modelled source of photons.

At the boundary, on which the radiation from the source is incident, the boundary condition for Eq. (1.1) is chosen as the total reflection condition. It is also assumed that the photons are free to escape from the calculation domain through the rest boundaries.

2.2 Solution approach

The source singularity makes it impossible to use the finite difference approximations directly for the solution of the considered problem. Therefore, the desired solution is presented as a sum of three functions

$$\Psi(\vec{r}, \vec{\Omega}, t) = \Phi_0(\vec{r}, \vec{\Omega}, t) + \Phi_1(\vec{r}, \vec{\Omega}, t) + \Phi(\vec{r}, \vec{\Omega}, t), \quad (2.1)$$

where the functions $\Phi_0(\vec{r}, \vec{\Omega}, t)$, $\Phi_1(\vec{r}, \vec{\Omega}, t)$ describe the flux density of non-scattered and singly scattered photons, and the function $\Phi(\vec{r}, \vec{\Omega}, t)$ describes the flux density of photons that undergo two or more scattering events. Substituting the representation (2.1) into Eq. (1.1) and using the additivity property of the transfer

equation, we arrive at the boundary problem for each component of the solution.

For the non-scattered component $\Phi_0(\vec{r}, \vec{\Omega}, t)$

$$\frac{1}{v} \frac{\partial \Phi_0}{\partial t} + \hat{L} \Phi_0 = Q, \quad \Phi_0(\vec{r}, \vec{\Omega}, t) \Big|_{\vec{\Omega} \cdot \vec{n} < 0} = 0. \quad (2.2)$$

For the singly scattered component $\Phi_1(\vec{r}, \vec{\Omega}, t)$

$$\frac{1}{v} \frac{\partial \Phi_1}{\partial t} + \hat{L} \Phi_1 = \hat{S} \Phi_0, \quad \Phi_1(\vec{r}, \vec{\Omega}, t) \Big|_{\vec{\Omega} \cdot \vec{n} < 0} = 0. \quad (2.3)$$

For the component allowing for two or more scattering events $\Phi(\vec{r}, \vec{\Omega}, t)$

$$\frac{1}{v} \frac{\partial \Phi}{\partial t} + \hat{L} \Phi = \hat{S} \Phi + \hat{S} \Phi_1, \quad \Phi(\vec{r}, \vec{\Omega}, t) \Big|_{\vec{\Omega} \cdot \vec{n} < 0} = 0. \quad (2.4)$$

The solution of the problem (2.2) is found analytically using the technique of generalised functions. Similarly, the right-hand side $\hat{S} \Phi_0$ in Eq. (2.3) and the solution of this equation are found.

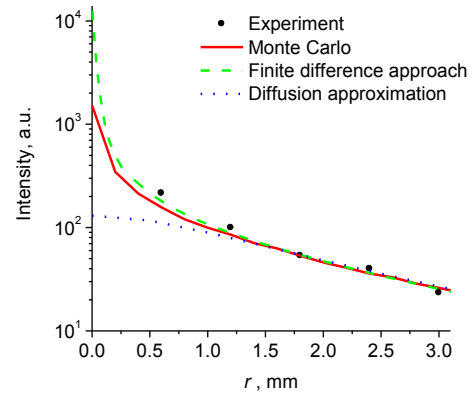
The solution of Eq. (2.4) has already no singularity and, therefore, is found using the integro-interpolational finite difference scheme. The system of finite-difference equations approximating Eq. (2.4) is solved using the iteration process for the collisions. The integrals (the operator S) in the right-hand side of Eq. (2.4) are replaced with quadrature sums.

Note, that since the problem is non-stationary, great computational load is required for time intervals (steps). To reduce the computation time one can use parallel algorithms. The parallelisation of the calculations can be implemented by means of the widely used method of spatial subdomains.

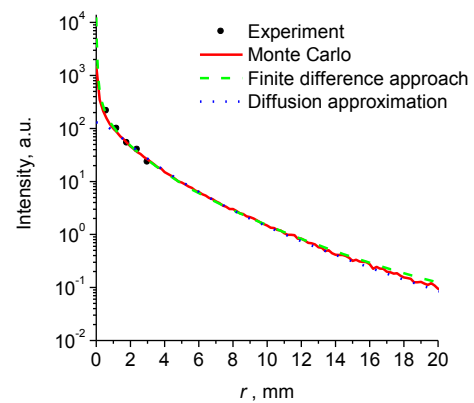
If the problem is stationary, then the first term in the left-hand side of Eqs. (1.1), (2.2)-(2.4) is absent, and the algorithm of solution corresponds to a single time step in the problem (1.1).

3 Comparison of finite difference methods with Monte Carlo method

The algorithm presented above was implemented in the M.V. Keldysh Institute of Applied Mathematics, Russian Academy of Sciences, in the form of the software package Raduga-5.1(P) for the stationary problem [9-11] and Raduga-5.2(P) for the non-stationary one [12-15]. Ref. [11] presents the comparison of the spatial distribution of the scattered intensity calculated using the finite difference method and the Monte Carlo methods for the stationary problem (Fig. 2a, b).



a



b

Fig. 2 Scattered intensity versus the distance r from the source, experimentally measured in a biotissue phantom and calculated using the finite difference method (RADUGA-5.1(P) software), the Monte Carlo method, and the diffusion approximation within the ranges $r = 0-3$ mm (a) and $r = 0-20$ mm (b).

Fig. 2 shows that the results of Monte Carlo simulation and the solution of the transfer equation using the finite difference method agree well with the results of experiment in the biotissue model, whereas the solution of diffusion equation is unsatisfactory in the vicinity of the source (Fig. 2a), which gives rise to large errors in attempts to reconstruct the medium properties using the solution of RTE in the diffusion approximation.

Besides that, at large distances from the source the Monte Carlo solution demonstrates oscillations (Fig. 2,b), which are absent in the finite-difference solution of the RTE. These oscillations are due to insufficient number of photon trajectories contributing to the signal.

The comparison of finite-difference and Monte Carlo solutions of a non-stationary problem was presented in Ref. [15]. Fig. 3 shows the results for a delta-pulse, backscattered by the medium, calculated using these methods. One can see good agreement of the results.

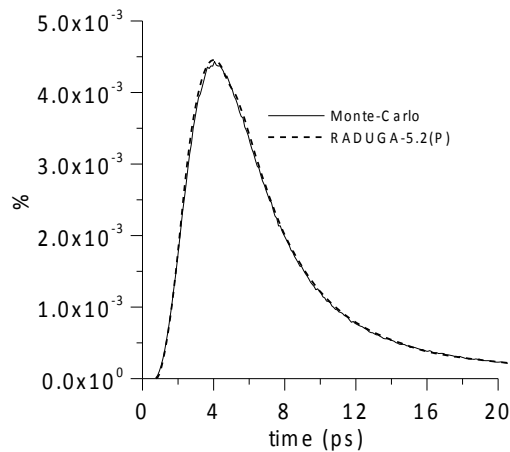


Fig. 3 Comparison of numerical simulations of the medium response to a delta-pulse from a monodirectional point source using the finite difference method (RADUGA-5.2(P) software) and the Monte Carlo method.

To implement the finite difference method, 49 processors 2xAMD Opteron 248 (2.2 GHz) were used; one calculation took 25 minutes. For the validation of the developed algorithm and assessment of its efficiency, the solution of the same problem was obtained using the Monte Carlo method with 10^9 trajectories. The Monte Carlo calculation took 10 minutes using 100 Intel Xeon processors (2.8 GHz), so that the both approaches required comparable computational time expenses.

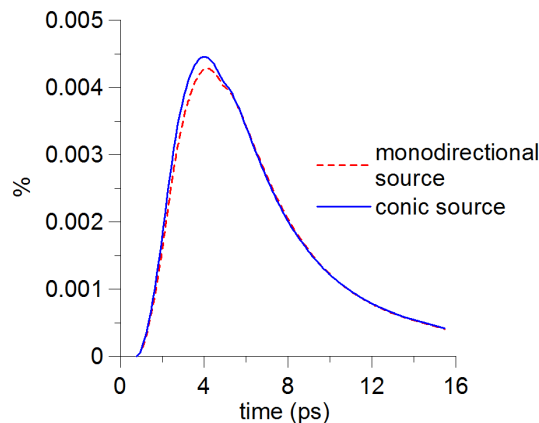


Fig. 4 Comparison of numerical simulations of the response of the medium, modelling human skin with the glucose content 500 mg/dl, to the delta-pulse from monodirectional point and conical (focused) radiation sources.

One more advantage of the considered finite difference approach is the possibility to choose the light beam geometry. In most problems, they consider point monodirectional beams, and the solution for a beam with complex geometry in the case when all rays are similarly directed can be obtained, e.g., by the convolution of the solution for a monodirectional point beam with the beam function (see, e.g., [7]). However,

such approach is not applicable to focused beams, since in this case the photons have different initial direction of propagation. In Refs. [12, 13] the finite difference solution of the non-stationary RTE was implemented for the conical-shaped beam. The results for the monodirectional beam and the conical one are compared in Fig. 4 [13].

4 Application of finite difference methods of solving RTE to the problems of optical biomedical diagnostics

The main goal of Refs. [13, 15] was to use numerical simulation for studying the efficiency of time-resolved optical probing of skin aimed at the assessment of glucose content in blood. The typical responses for skin probing with a delta-pulse (the probing geometry is presented in Fig. 5a), the glucose concentration being 0 and 500 mg/dl, are presented in Fig. 5b. It is shown that the difference in the maxima of the recorded pulses amounts to 13%, which is sufficient for *in vitro* measurements. However, for the *in vivo* probing this difference can be masked by the typical spread of the skin optical characteristics.

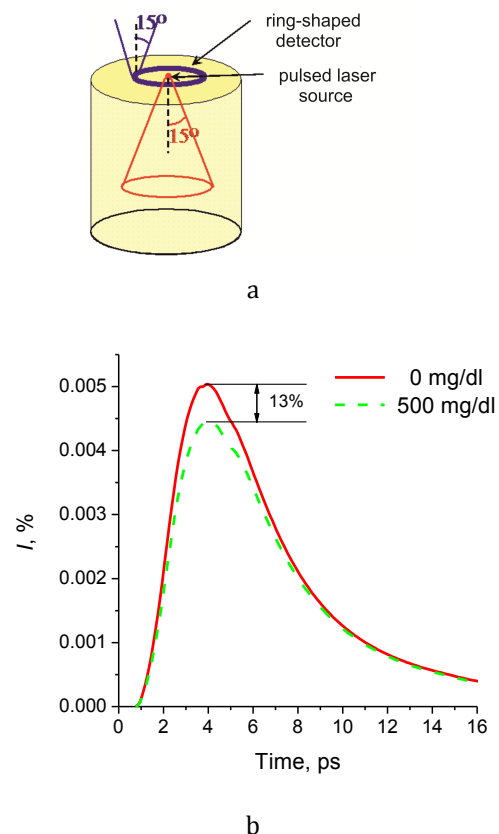


Fig. 5 (a) Geometry of probing a homogeneous biotissue phantom aimed at the blood sugar measurement by means of the pulsed conical (focused) laser radiation and ring detector. (b) Numerical simulation of the human skin model response to the delta-pulse of conical (focused) light source. The curves correspond to the glucose content of 0 and 500 mg/dl.

In Refs. [16, 17] the results of numerical simulation using the Monte Carlo method and finite difference methods were used to develop an approach to the solution of inverse problem of blood vessels diagnostics with the application of a neuron network. Fig. 6a [16] schematically shows the biotissue phantom with vessels, and Fig. 6b illustrates the result of simulating the continuous-wave laser radiation, reflected from this phantom. It is worth nothing that the signal I_V from the phantom with a vessel enters the denominator of the function F , shown in Fig. 6b, which provides a clearer result for the vascular diagnostics.

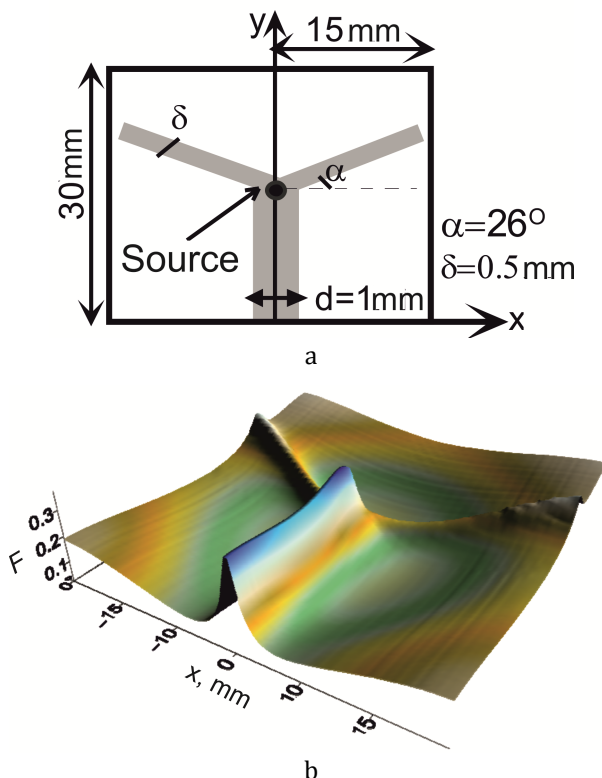


Fig. 6 (a) Schematic top view of biotissue phantom with a branching blood vessel. The phantom thickness is 20 mm, the blood vessel axis is at the depth 1 mm. (b) Surface distribution of the radiation intensity diffusely reflected from the considered phantom. The 2D distribution of $F = \ln(I_0 / I_V)$ is presented, where I_V and I_0 is the diffuse reflected signal from the phantom with a branching vessel and from a similar phantom without a vessel, respectively.

The purpose of Ref. [17] was to develop an approach to the automated determination of the diameter and depth of location of blood vessel in the model biotissue. For this aim using the numerical methods, the signals of spatially resolved reflectometry were simulated for different positions and diameters of the vessel. The results were used as a learning sample for the neuron network with the number of neurons from 2 to 5, depending on the desired accuracy of reconstruction. It was shown that the relative error of determining the diameter and the location depth of the vessel by means of the neuron network does not exceed 2% for the considered model of a cylindrical vessel in homogeneous medium.

5 Conclusion

The finite difference methods of solving the radiative transfer equation (RTE) provide an efficient tool for the solution of the problem of optical-range radiation propagation through biological tissues, which allows the solution of both direct and the inverse problem of optical diagnostics. The comparison of finite difference methods with Monte Carlo simulations (MC) showed good agreement of the obtained results, the finite difference methods providing smoother curves as compared to the MC method. The finite difference methods have been applied to the problems of optical biomedical diagnostics for the solution of both stationary and non-stationary RTE for different beam geometries.

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