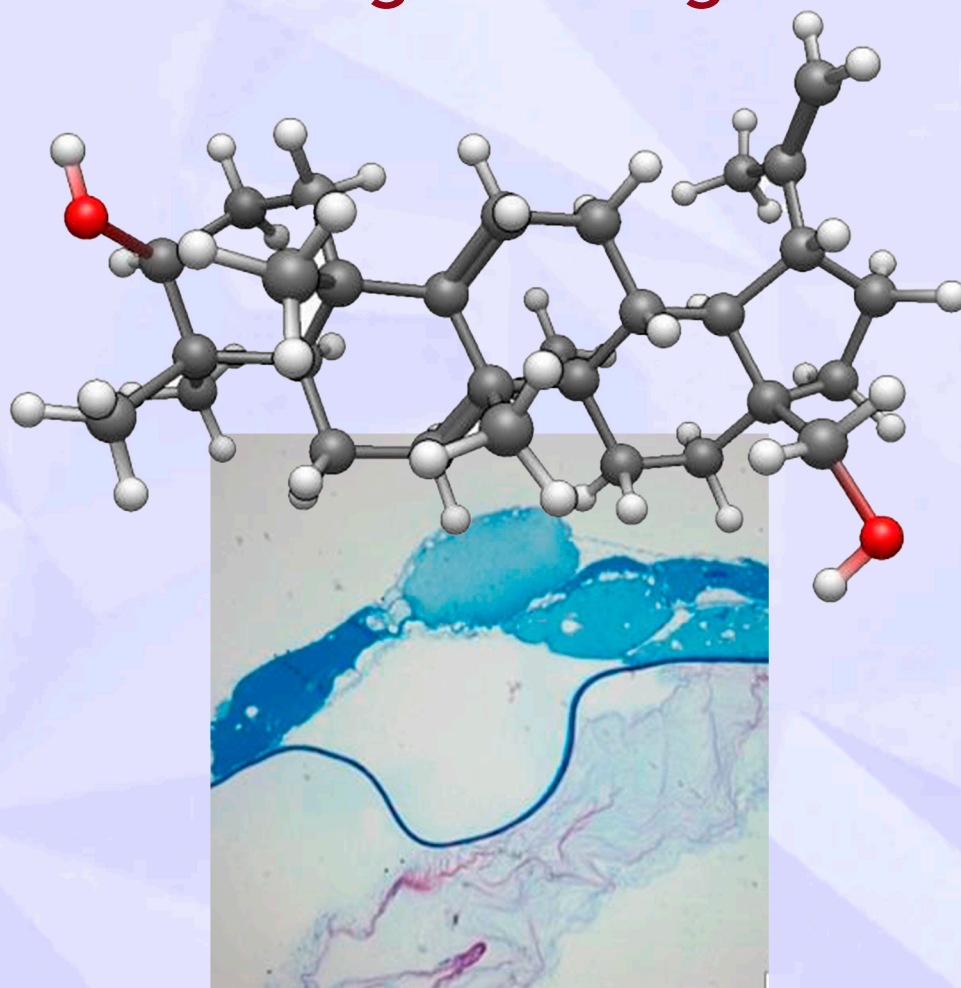


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Olga I. Baum et al., Laboratory Modeling Techniques for Primary and Secondary Cataracts: Methodology and Analysis, 020307, Fig. 2b.

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Singlet Oxygen Detection: A Review of Recent Advances

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Abstract. As part of the search for and development of an optimal solution related to singlet oxygen ($^1\text{O}_2$) detection, an analytical review of research in this direction was carried out. $^1\text{O}_2$ is one of the key participants in many biological processes, so its detection and numerical estimation are the subject of a close study by many research groups. To date, several approaches to the optical detection of $^1\text{O}_2$ have been proposed, in most cases based on the registration of $^1\text{O}_2$ luminescence in the near-infrared range of the spectrum. The review also presents various fluorescence probes, chemical traps and several other tools that are also used for $^1\text{O}_2$ detection.

Keywords: singlet oxygen; luminescence detection; probes; chemical traps; electron paramagnetic resonance spectroscopy (EPR); quantitative PCR (qPCR).

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1 Introduction

Today, one of the developing fields of biology is redox biology, which aims to study reactive oxygen species (ROS). Singlet oxygen ($^1\text{O}_2$) is one of the most reactive forms of ROS. $^1\text{O}_2$, discovered and named by Herzberg [1] in 1934, is a product of the activation of the triplet state of molecular oxygen ($^3\text{O}_2$). It is generated through photochemical reactions, for example, when light is absorbed by photosensitizers (PS) or during metabolic processes in mitochondria and chloroplasts. Its high reactivity allows it to interact with lipids, proteins, and nucleic acids, causing their oxidation and damage. At the same time, $^1\text{O}_2$ is involved in the regulation of cellular signaling pathways, such as apoptosis and autophagy, highlighting its dual role in the cell [2–3].

Particular interest in $^1\text{O}_2$ is due to its key role in photodynamic therapy (PDT) of malignant tumors, where it is used to selectively destroy cancer cells [4–6]. Selectively means that photosensitizer is accumulated in tumor tissue and does not affect healthy tissue in a destructive manner. Upon irradiation with light of a specific wavelength, the PS generates $^1\text{O}_2$, which induces localized oxidative damage, leading to cancer cell death via apoptosis, necrosis, or vascular shutdown. In addition, its antimicrobial properties have applications in the development of new disinfection methods [7]. In industry and organic synthesis, $^1\text{O}_2$ is used for the selective oxidation of complex molecules, which allows

the creation of new materials and pharmaceutical compounds. However, despite its obvious benefits, its use is associated with a number of challenges and limitations.

The main difficulty lies in the generation of $^1\text{O}_2$. In nature, it can be produced by ultraviolet radiation, during photosynthesis and by some enzymes. In laboratory and industrial conditions, it is produced with the help of photosensitizers – compounds capable of transferring energy to molecular oxygen, converting it to the singlet state – or directly, at excitation wavelengths of triplet oxygen [8]. However, the efficiency of such processes depends on many factors, including the type of sensitizer, the wavelength of light used, and the environment in which the reaction takes place [7]. The detection of $^1\text{O}_2$ is no less challenging. Because of its short lifetime (nanoseconds to microseconds, depending on the medium) and high chemical activity, direct detection is difficult. Thus, despite the high interest in $^1\text{O}_2$, issues related to its generation, stability, and detection remain unresolved. Modern research is aimed at developing more efficient methods for its generation and accurate detection, which will open new opportunities for the application of this unique compound in medicine, biotechnology and industry.

This review discusses methods for its detection and the existing problems that hinder their effective use in various fields of science and techniques.

2 Singlet Oxygen Luminescence Detection

2.1 Historical Overview

After its formation, a molecule of $^1\text{O}_2$ can undergo nonradiative decay, oxidize surrounding biomolecules or undergo radiative decay at around 1270 nm. The detection of this near-infrared (NIR) emission through time-resolved techniques is a widely employed approach for assessing the lifetimes and quantum yields of $^1\text{O}_2$.

$^1\text{O}_2$ emits ultra-weak luminescence at around 1270 nm as it relaxes from its excited state to the ground state. This radiative transition is spin-forbidden, resulting in a very low quantum yield (typically 10^{-6} to 10^{-8}) and, consequently, low emission intensity [9]. The lifetime of $^1\text{O}_2$ varies with the surrounding medium, ranging from tens of microseconds in the gas phase to less than a microsecond in biological environments. Given the possible concentrations of $^1\text{O}_2$ generated [10], the absolute intensity of its luminescence is extremely low, typically in the femtowatt (10^{-15} W) to picowatt (10^{-12} W) range. At the same time, even lower values should be expected with direct optical generation.

The first studies in the field of time-resolved detection of $^1\text{O}_2$ based on the luminescence properties were undertaken by Krasnovsky's group back in 1976 [11]. The approach developed in this work is by now considered to be the standard approach for the study of $^1\text{O}_2$ photochemistry. $^1\text{O}_2$ phosphorescence was studied in pigment solutions containing no hydrogen atoms, and spectrometers with mechanical phosphoroscopes and a photodetector system based on cooled photomultiplier tubes were used for its registration. The excitation, emission, and luminescence lifetime spectra of $^1\text{O}_2$ were studied [12] (see Fig. 1).

Over the years, several teams have attempted to measure $^1\text{O}_2$ levels under conditions that approximate physiological conditions. For example, B. Roeder et al. demonstrated time-resolved emission experiments to detect $^1\text{O}_2$ luminescence in a suspension of haemolysed erythrocytes [13]. However, separating the useful signal from the strong background emission required a careful approach in the selection of the sensitiser, optimisation of the detection system, use of D_2O as solvent and sodium azide as a quencher, and variation of the O_2 concentration. Thus, the demonstrated approach did not allow adequate measurement of $^1\text{O}_2$ under physiological experimental conditions *in vitro* or *in vivo*.

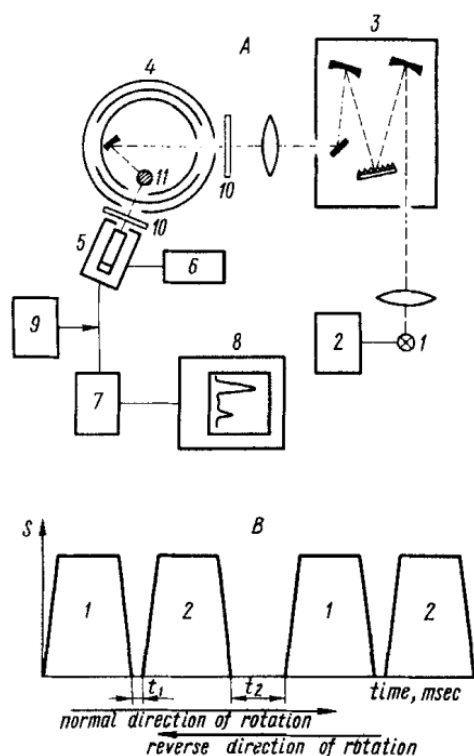


Fig. 1 (A) Block-scheme of the apparatus for measuring excitation spectra of the $^1\text{O}_2$ luminescence: (1) Xe lamp 1000 W, (2) power supply, (3) grating monochromator, (4) phosphoroscope, (5) photomultiplier, (6) high voltage supply, (7) direct current amplifier, (8) chart recorder, (9) oscilloscope (to monitor the speed of rotation), (10) light filters, (11) sample holder. (B) Sequence and time course of opening and closing the phosphoroscope holes: (1) the phase of excitation, (2) the phase of luminescence measurement. t_1 and t_2 are dark intervals, S is the area of the holes opened for the passing of light [12]. Reproduced with permission of John Wiley and Sons.

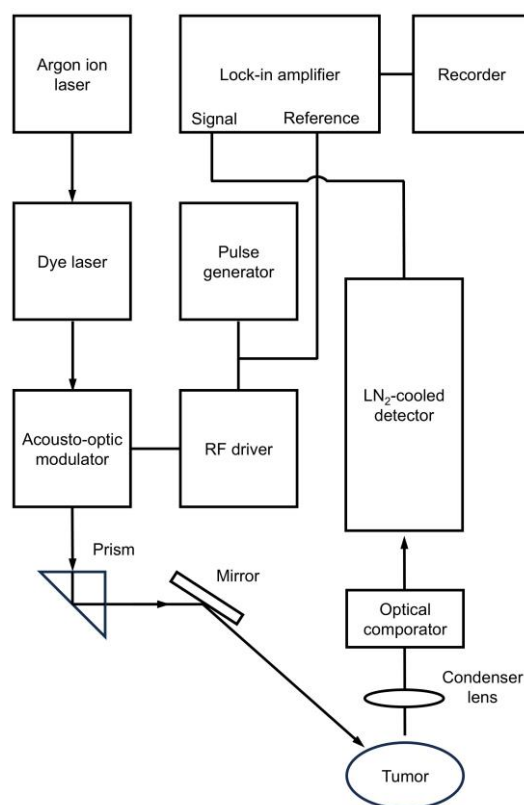


Fig. 2 Schematic diagram of apparatus used to detect $^1\text{O}_2$ emission from a laser-irradiated SMT-F tumor implanted subcutaneously in a type-DBA/2 mouse. Mouse had been previously injected intraperitoneally with Photofrin II at a level of 50 mg/kg. Incident laser power was typically 20 mW and the wavelength set at 630 nm. Adopted from Ref. [14].

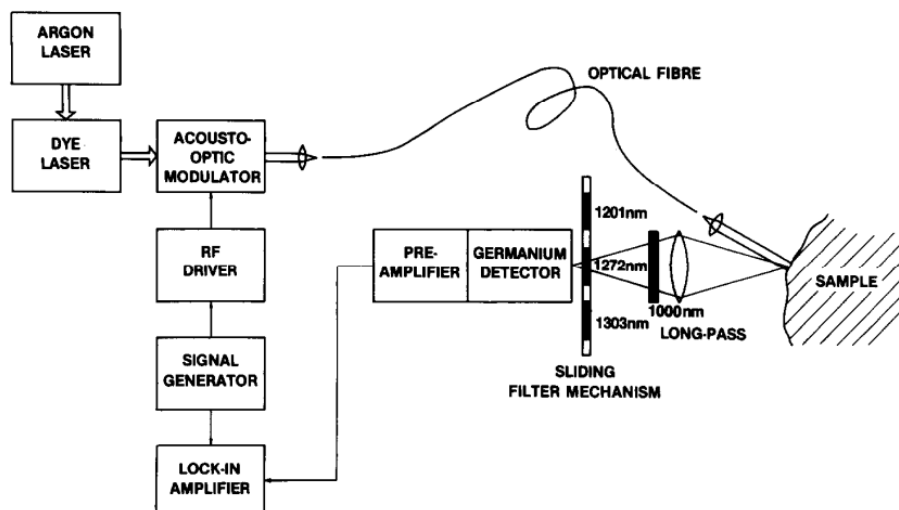


Fig. 3 Schematic diagram of apparatus for the detection of $^1\text{O}_2$ luminescence *in vivo*. The sliding filter mechanism facilitates the quadrature detection method and allows rough verification of the luminescence spectrum. For *in vitro* experiments, a quartz cuvette (1 cm) was used, and samples were irradiated through the side face [15]. Reproduced with permission of Elsevier.

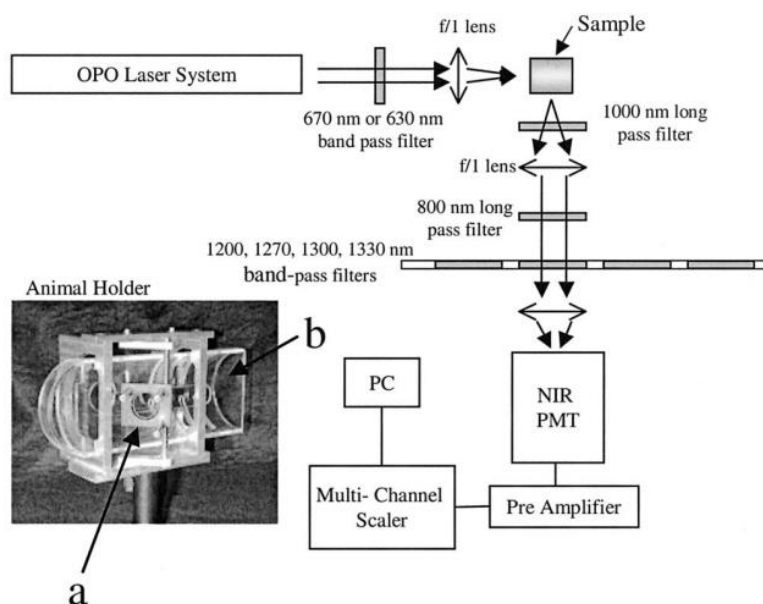


Fig. 4 Schematic of the experimental system used for $^1\text{O}_2$ luminescence detection. Inset: purpose-built animal holder used for *in vivo* experiments. Excitation light was delivered through the circular port (a), and measurements were made through a second port at 90° (not seen). The animal was held in the hemispheric cylinder (b) [16]. Reproduced with permission of John Wiley and Sons.

The multiple shortening of the lifetime of $^1\text{O}_2$ in cells and tissues (Figs. 2–3), caused by its rapid quenching by biomolecules, and the lack of sufficiently sensitive detectors in the near-infrared wavelength range have long prevented the development of an approach to measuring $^1\text{O}_2$ levels under physiological conditions that would give consistent results [14, 15].

In 2002, a group of researchers led by B. S. Wilson proposed the use of near-infrared luminescence measurement during photodynamic therapy as a valuable tool for direct dosimetric assessment of changes in $^1\text{O}_2$ levels [16]. The experimental setup, which is shown in

Fig. 4, included the use of a tunable pulsed Nd:YAG laser, 4 bandpass filters with maximum transmission in the range of 1200, 1270, 1300 and 1330 nm and a photodetector based on the use of a liquid nitrogen cooled photomultiplier tube, which allows extremely sensitive detection in the range of 1200–1330 nm and has a fast response time. The authors performed experiments with photosensitiser solutions, *in vitro* studies using cell suspension, and *in vivo* studies on Wistar rats with generation and measurement of $^1\text{O}_2$ in skin and liver surface areas.

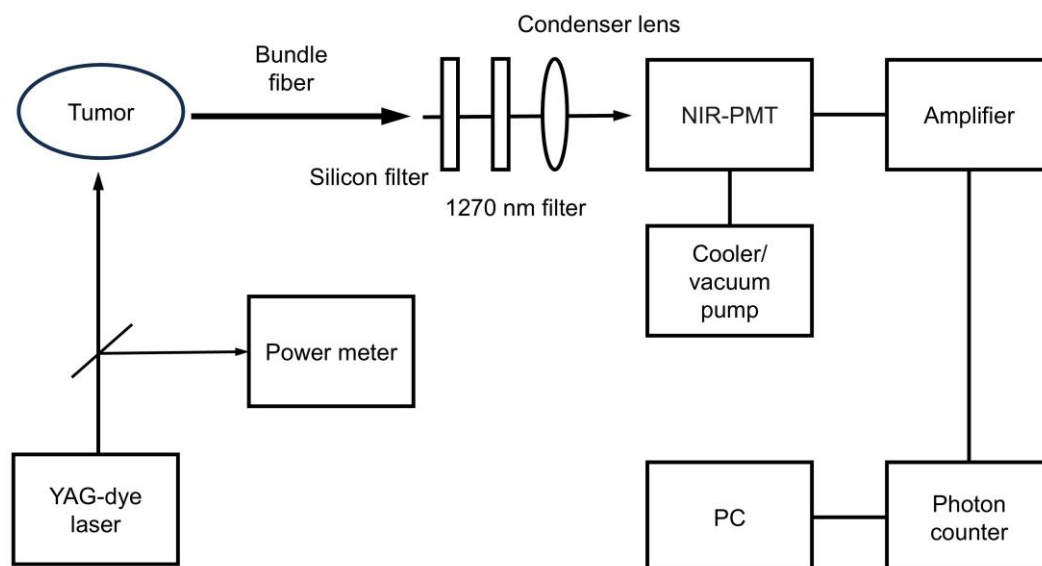


Fig. 5 Schematic drawing of the experimental setup for *in vitro* and *in vivo* monitoring of $^1\text{O}_2$. Adapted from Ref. [20].

The study showed that the application of the approach developed by the authors provided convincing evidence that $^1\text{O}_2$ luminescence from an intracellular photosensitizer could be detected and quantified in cell suspension in an H_2O -based medium and in tissues of laboratory animals *in vivo*. Later, the proposed approach was repeatedly applied in studies of $^1\text{O}_2$ generation efficiency in photodynamic therapy of implanted murine tumours sensitized with ATX-S10 [17], killing OCI-AML5 leukaemia cells treated with aminolevulinic acid (ALA) induced protoporphyrin IX (PpIX) [18, 19], and in the evaluation of PDT efficiency in an experimental model of glioma sensitised with methylene blue (see Fig. 5) [20].

Li et al. used an approach based on the use of photomultiplier tubes with high sensitivity in the NIR range to measure the time-resolved luminescence of $^1\text{O}_2$ and an optical filtering system to increase the sensitivity of $^1\text{O}_2$ detection [21]. A bifurcated optical probe was used to deliver pulsed laser light and collect $^1\text{O}_2$ emission, which facilitates the application of the system in *in vivo* experiments. Since the detected emission includes both the $^1\text{O}_2$ luminescence signal and other possible NIR emissions such as photosensitizer fluorescence, tissue phosphorescence and autofluorescence, etc., for spectral separation of $^1\text{O}_2$ radiation from interference, the recorded near-IR radiation was passed through a series of three narrow-band filters with a bandwidth of 15 nm and central wavelengths of 1220, 1270, and 1315 nm. The peak of $^1\text{O}_2$ luminescence was centred in the 1270 ± 10 nm wavelength region. Two filters centred at 1220 and 1315 nm provided background emission measurements that did not contain $^1\text{O}_2$ emission. Data were recorded by a thermoelectrically cooled photomultiplier tube operating in photon counting mode, after which the signal was amplified and processed by a data acquisition system. The authors tested their $^1\text{O}_2$ detection method on rat tumours during PDT, using the prostate cancer cell line R3327-MatLyLu as an animal

model. The $^1\text{O}_2$ measurement sensor was placed 1.5 mm above the skin of the animals. The study protocol included recording $^1\text{O}_2$ emission before administration of the photosensitizer, just before the start of therapeutic light irradiation after the photosensitizer had spread systemically during a defined period of its incubation, several times during the therapeutic irradiation and at the end of light irradiation. The authors observed tumour regression that correlated with the measured level of $^1\text{O}_2$ production. The results of the study demonstrated a moderate signal-to-noise ratio and established a clear relationship between $^1\text{O}_2$ production and tumour shrinkage.

2.2 Recent Advances

However, the *in vivo* measurement of $^1\text{O}_2$ luminescence using photomultiplier tubes presents a number of problems since this type of photodetector has low efficiency and is highly sensitive to noise and external illumination, which prevents effective detection of luminescence in biological tissues with low emission and short lifetime (<1 ms). To overcome this problem, some teams of authors have turned to new technologies based on the use of superconducting single-photon detectors, which offer high sensitivity in the IR region, picosecond time resolution and low dark noise. The group of Hadfield et al. [22] proposed the first application of superconducting nanowire single-photon detectors for $^1\text{O}_2$ detection. In their study, $^1\text{O}_2$ emission was recorded using time-resolved and spectrally filtered measurements performed in a solution of Rose Bengal (RB). The study demonstrated the quenching of $^1\text{O}_2$ luminescence by sodium azide. Subsequently, bovine serum albumin (BSA) was used to mimic a biological medium containing the protein; with increasing BSA concentration, the evolution of the RB triplet lifetime and singlet state of $^1\text{O}_2$ were successfully observed. As expected, the lifetime of the RB triplet state increased

markedly upon binding to the protein, which is probably due to the shielding of the photosensitizer from the diffusion of oxygen molecules with which the triplet state can react. A decrease in the lifetime of $^1\text{O}_2$ was also observed in the studies performed and was attributed to the quenching of $^1\text{O}_2$ luminescence emission by the protein. The authors also demonstrated an optical fibre delivery and data acquisition scheme, which is particularly important in the field of $^1\text{O}_2$ detection for preclinical and clinical *in vivo* applications, which is shown in Fig. 6.

Alternative low-cost approaches to $^1\text{O}_2$ detection have been proposed in the work [23]. The authors developed a tool for measuring $^1\text{O}_2$ luminescence using an InGaAs avalanche photodiode and simple electronics. This approach was tested in organic solvents such as tetrachloromethane (CCl_4), ethanol, and DMSO. The authors have demonstrated spectrally resolved measurements of $^1\text{O}_2$ luminescence in CCl_4 using the developed setup, showing high complementarity with literature data on $^1\text{O}_2$ luminescence. The authors propose to apply the setup to evaluate the efficacy of new photosensitizers as well as for $^1\text{O}_2$ dosimetry during PDT, but for the latter application, additional work is needed to improve the sensitivity of the detector in order to apply it in real systems.

Since the biological environment is characterized by high heterogeneity, the registration of time-resolved $^1\text{O}_2$ luminescence does not always provide the desired result, as it may represent an average value for many very different deactivation processes. In this regard, the development of spatially resolved detection methods is of interest. The first steps in this field were made by the group of Ogilby et al. [24–26]. Using wide-field microscopy, they were able to detect the luminescence of $^1\text{O}_2$ in neurons produced by the photosensitizer TMPyP and obtained data on the typical distances over which $^1\text{O}_2$ diffuses inside cells. In the detection system developed by this group, the luminescence of the samples is collected by a microscope objective and passed through the same dichroic optics used to reflect Xe-lamp light. Spectral discrimination of $^1\text{O}_2$ phosphorescence in the near-IR is achieved using an interference filter centered at 787 nm, after which the emission is fed to a custom-built detector with a linear array of InGaAs photodiodes operating at -100°C . The detector contains 512 elements (pixels), each with a size of $50 \times 50 \mu\text{m}$. The spatial resolution available in this experiment is determined by a combination of the size of the detector array elements and the magnification of the objective lens. For data recorded with a $20\times$ objective lens, the authors obtained a resolution of up to $2.5 \mu\text{m}$.

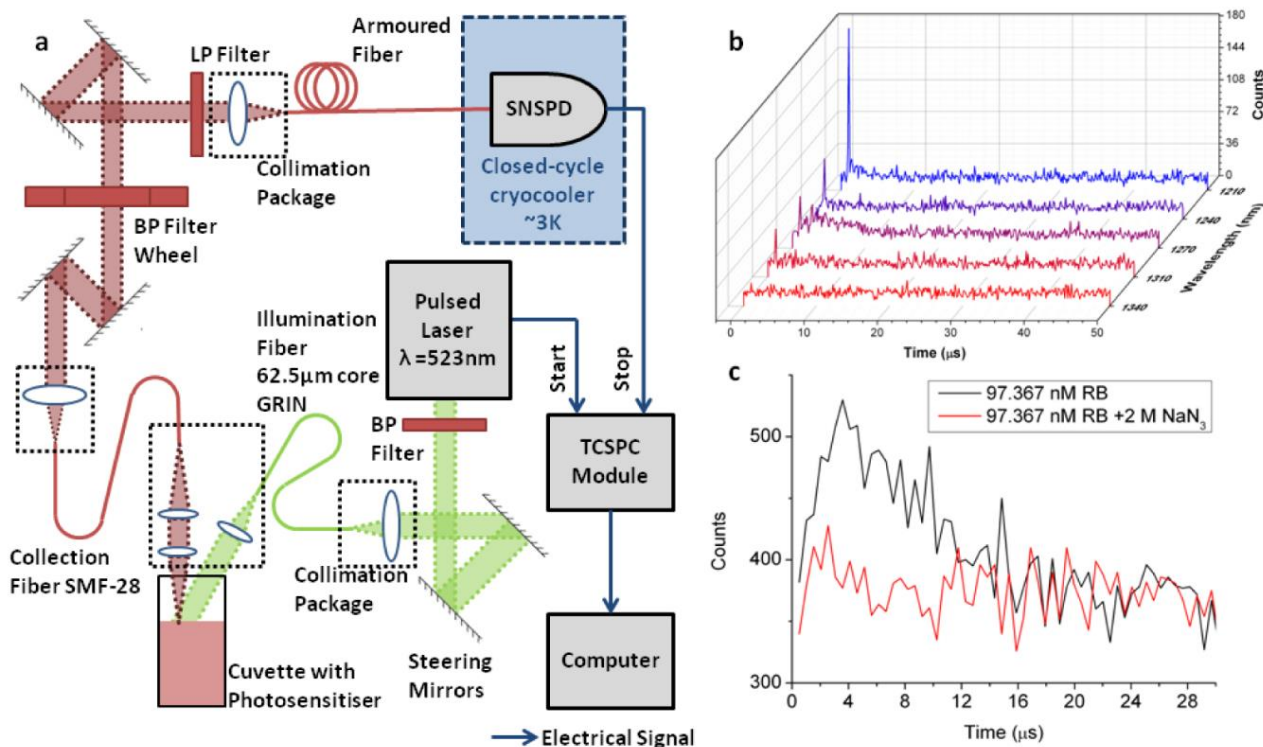


Fig. 6 (a) Fiber-based delivery and collection for $^1\text{O}_2$ luminescence detection: schematic of the experimental setup, (b) luminescence time histograms in RB (97.367 nM) for the different bandpass filters (30 min acquisition time, $0.128 \mu\text{s}$ bin size), (c) quenching with sodium azide [60 min acquisition time, $0.512 \mu\text{s}$ bin size] [22]. Optica Publishing Group Open Access Publishing Agreement.

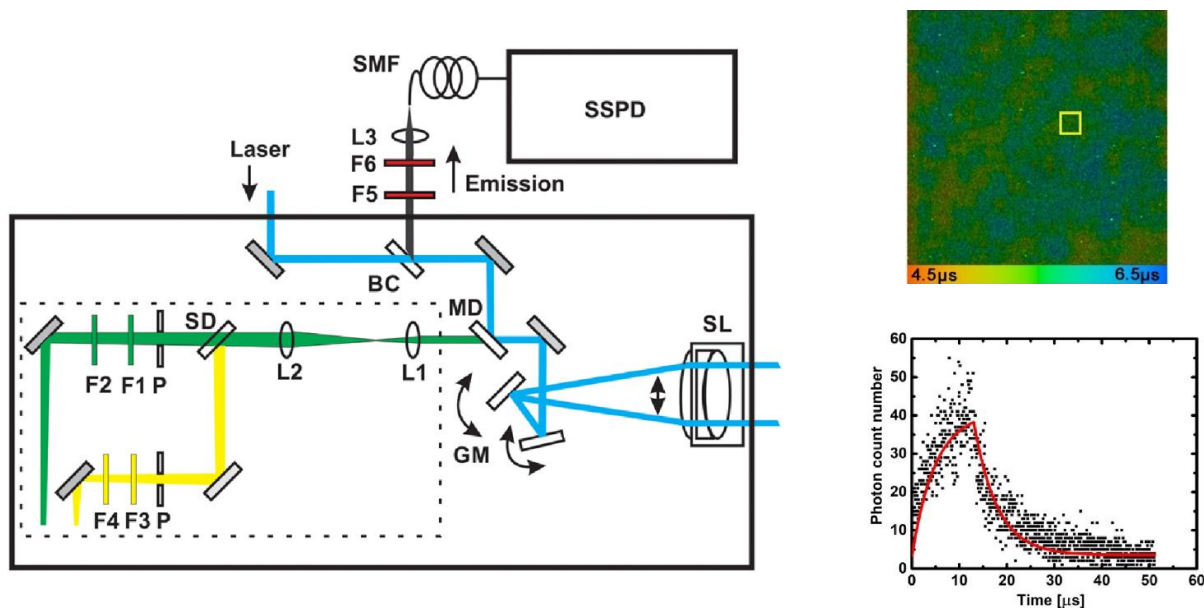


Fig. 7 Left part: Schematic of the experimental setup for $^1\text{O}_2$ measurements. SSPD: superconducting single-photon detector; SMF: single-mode fiber; BC: beam combiner 810LP; P: pinhole; F1, F3: long-pass filters; F2, F4: bandpass filters; F5: long-pass filter 830LP; F6: bandpass filter 1260/30. GM: galvo-mirrors; SL: scanning lens; MD: main dichroic mirror. The dashed line shows a part of the scanner that may be used for confocal detection of fluorescence from a sample. Right part (top): phosphorescence lifetime image (256×256 pixels) of $^1\text{O}_2$ in the solution of Ce6. The full scanned field corresponds to $200 \mu\text{m}$. Right part (bottom): time-resolved kinetics of the phosphorescence in the selected spot, marked with a square on the image. Excitation wavelength: 405 nm. Collection time: 600 s [27]. Reproduced with permission of Optica Publishing Group.

Another example of the study of $^1\text{O}_2$ distribution in cell culture and time-resolved measurement of its luminescence is the work of Shcheslavskiy's group [27], which presents a system based on a superconducting single-photon detector coupled to a confocal scanner modified for NIR measurements. The recording of the $^1\text{O}_2$ phosphorescence signal at 1270 nm was performed using time-correlated single photon counting (TCSPC). The system was verified by performing experiments both on solutions of photosensitizers frequently used in photodynamic therapy – Cle6 and methylene blue – and on HeLa cells (Fig. 7).

The paper demonstrates the ability of the system to record the lifetime distribution of $^1\text{O}_2$ luminescence over an image; however, the data acquisition time in this system is rather long due to the low quantum yield of $^1\text{O}_2$ luminescence and the rapid quenching of $^1\text{O}_2$ in the cells. The authors propose to use a fluorescence image of an object to find the region of interest and then record $^1\text{O}_2$ luminescence kinetics only from specific points in the image to circumvent this problem of long data acquisition time.

The research team of Hackbarth et al., in their paper [28], studied the $^1\text{O}_2$ luminescence kinetics in tumour cells of a mouse model *in vivo*. The time-correlated multiple photon counting (TCMPC) method in the NIR region with central wavelengths of 1200, 1270 and 1340 nm was used to record $^1\text{O}_2$ phosphorescence *in vivo*. The authors studied the dynamics of changes in $^1\text{O}_2$ phosphorescence during PDT of a cancer tumour model obtained by subcutaneously implanting the S180

sarcoma cell line into laboratory animals. The study showed a very weak $^1\text{O}_2$ phosphorescence signal *in vivo* in contradiction to the parallel results obtained on cell culture *in vitro*, which they believe may be due to oxygen depletion even at moderate (on the scale of PDT) illumination intensity, shortly after the start of illumination.

Although the new technologies being developed look extremely promising, optical detection methods suffer from a low level of $^1\text{O}_2$ luminescence emission and its rapid attenuation, so the quantitative detection of very small concentrations of $^1\text{O}_2$, especially with direct optical generation [8], is currently very difficult, which is especially critical when conducting *in vivo* studies.

3 Singlet Oxygen Detection Using Probes

Various probes are also used to detect $^1\text{O}_2$, which can be divided into three main groups: absorption-based probes, photoluminescent probes and chemiluminescent probes [29]. Probes for $^1\text{O}_2$ detection must meet a number of requirements, including sensitivity, fast response time, selectivity with respect to other ROS. Moreover, reversibility may be advantageous in specific scenarios (e.g., real-time continuous monitoring of $^1\text{O}_2$ dynamics), but it should not necessarily be prioritized over robustness and detection limits. Additionally, water-solubility is a practical necessity for biological studies, as many $^1\text{O}_2$ reactions occur in aqueous media. Probes used to detect $^1\text{O}_2$ in biological media must also meet additional conditions. They should have low cytotoxicity and sufficient dynamics to register rapid processes [26, 29, 30].

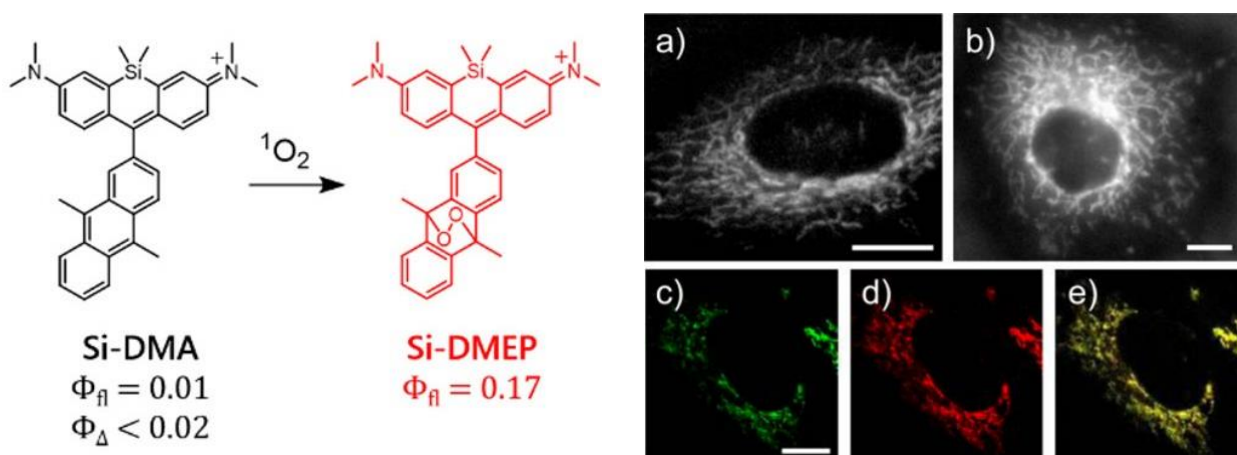


Fig. 8 Left part: chemical structures of Si-DMA, its peroxidized product (Si-DMEP). Right part: Example of HeLa cells stained with [Si-DMA] = (a) 20 and (b) 100 nM for 1 h. Colocalization test of Si-DMA in mitochondria (c–e). (c) MitoTracker Green and (d) [Si-DMA] = 100 nM. Clear colocalizations of two dyes are observed in the merged image (e), and the intensities of each image have been adjusted to obtain a clear picture. Scale bar = 10 μm (B) [31]. Reproduced with permission of American Chemical Society.

Majima et al. synthesized a new far-red fluorescence probe (excitation/emission maxima at 640/670 nm) composed of silicon-containing rhodamine and anthracene moieties, namely Si-DMA, as a chromophore and a $^1\text{O}_2$ reactive site, respectively [31]. In the presence of $^1\text{O}_2$, the fluorescence of Si-DMA increases 17 times due to endoperoxide formation at the anthracene moiety (Fig. 8). Among seven different ROS, Si-DMA is able to selectively detect the $^1\text{O}_2$. In addition, Si-DMA is able to visualize the real-time generation of $^1\text{O}_2$ from protoporphyrin IX in mitochondria with 5-aminolevulinic acid (5-ALA), a precursor of heme. This dye makes it possible to visualize $^1\text{O}_2$ generated during PDT with a spatial resolution of a single mitochondrial tubule.

Over the past few decades, the photoinduced electron transfer (PET) process has become widespread for the development of selective probes for $^1\text{O}_2$ detection. The anthracene link is commonly used as the central core of the fluorophore in the construction of PET-based probes due to its rapid reaction with $^1\text{O}_2$ [32]. The Singlet Oxygen Sensor Green (SOSG) is a widely recognized and commercially available $^1\text{O}_2$ detection probe. The SOSG molecule consists of two parts: a fluorophore and an anthracene-derived trapping moiety. In the absence of $^1\text{O}_2$, emission from the fluorophore is quenched by electron transfer from the adjacent trapping moiety. The indicator exhibits weak blue fluorescence with excitation/emission maxima at 372/395 and 393/416 nm. In an environment containing $^1\text{O}_2$, the trapping moiety reacts with $^1\text{O}_2$ and forms an endoperoxide (EP) anthracene, which is no longer an efficient intramolecular electron donor (see Fig. 9). Removing the quenching leads to the fluorophore's fluorescence at excitation/emission maxima of about 504/525 nm [33].

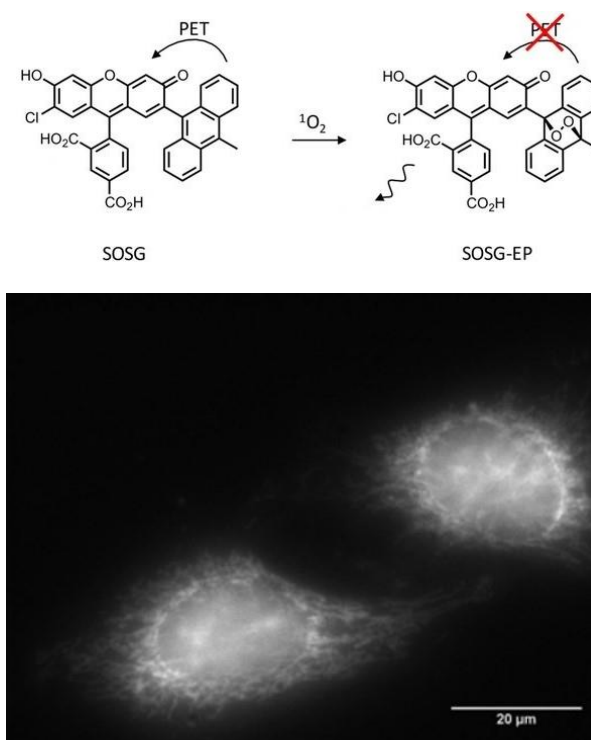


Fig. 9 Top part: chemical structure of SOSG and the formation of SOSG-EP upon interaction with $^1\text{O}_2$, leading to activation of fluorescence output. Bottom part: Image of two HeLa cells based on the fluorescence of SOSG. Data were recorded after 2 h incubation, in the dark, in our standard maintenance medium into which SOSG had been dissolved. The nuclei are visible as the large oval structures, and SOSG-containing filamentous structures in the cytoplasm clearly extend out to the cell membrane [33]. Reproduced with permission of John Wiley and Sons.

However, the ability of SOSG to penetrate cell membranes is still an open question [34]. Some studies have shown that SOSG can be absorbed by cells in stressful situations or in the absence of proteins. Work [35] demonstrates that different microbial species and cancer cells become fluorescent when exposed to SOSG under conditions which exclude the generation of $^1\text{O}_2$. Cells permeabilized with chlorhexidine or by heat exposure under anaerobic conditions exhibited SOSG fluorescence. A number of authors claim that this probe is not suitable for most biological purposes [36]. Also, when using SOSG in tissue slices, there is a slight increase in the fluorescence intensity during $^1\text{O}_2$ generation. One of these limitations is the short lifetime of $^1\text{O}_2$, which leads to a higher probability of alternative interactions occurring at a higher rate. This is due to the significant reaction rate constant and the relatively low operating concentration of SOSG. As a result, these interactions can weaken or completely prevent the reaction between $^1\text{O}_2$ and SOSG necessary for the formation of a fluorescent product [37]. To increase biocompatibility, SOSG is covalently linked to a polyacrylamide nanoparticle core using different architectures. NanoSOSG is easily absorbed by cells, and at the same time, the spectral characteristics do not change inside the cells, and the intracellularly generated $^1\text{O}_2$ probe reacts with an increase in fluorescence [38]. Also, the Aarhus Sensor Green (ASG) probe is an effective solution to the disadvantages of commercially available SOSG probes. Unlike SOSG, ASG includes fluorine atoms, effectively preventing self-photosensitization at physiological pH. This characteristic gives ASG a clear advantage over SOSG [39].

UV-Vis spectroscopy is also a widely used method for measuring $^1\text{O}_2$. Anthracene and benzofuran are well-known compounds with the ability to selectively absorb $^1\text{O}_2$ which is shown in Fig. 10.

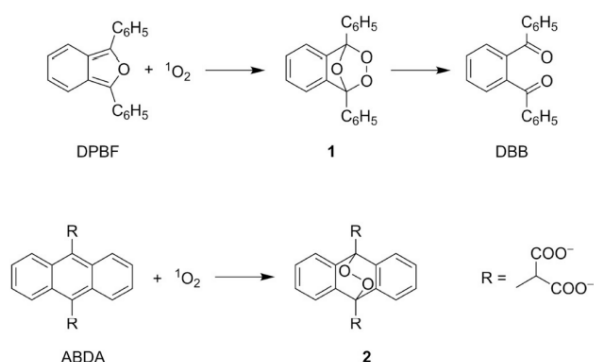


Fig. 10 Reaction of $^1\text{O}_2$ with DPBF (top) and ABDA (bottom) [40]. Creative Commons CC-BY.

When these aromatic molecules are oxidized by $^1\text{O}_2$, their absorption decreases, which allows a semi-quantitative assessment of the $^1\text{O}_2$ present. Although UV-Vis spectroscopy is less sensitive than fluorescence spectroscopy, it is less susceptible to distortion caused by impurities due to the absence of quenching [40]. For UV-

Vis spectroscopy, 1,3-diphenylisobenzofuran (DPBF) is one of the most commonly used probes for $^1\text{O}_2$ detection [41]. DPBF can also be used in fluorescence spectroscopy due to its strong fluorescence signal. However, DPBF is a water-insoluble probe, which significantly limits its application to biological media.

4 Other Possible Approaches

One of the detection methods is also the use of chemical “traps”, for example, histidine, to capture $^1\text{O}_2$ using imidazole rings to form a dioxygen complex. The decrease in the volume concentration of oxygen can be precisely controlled using an oxygen electrode (Clark electrode) [37, 42].

In addition, special spin traps have been developed that react with $^1\text{O}_2$ to form a stable nitroxide radical, which can be measured using electron paramagnetic resonance (EPR) spectroscopy [43]. The theoretical detection limit of EPR in the Bruker ELEXSYS E500 spectrometer has an approximate concentration sensitivity limit of 4.0×10^{-12} mol. However, it is worth noting that this method has certain limitations since various biomolecules can potentially interact with spin traps. Therefore, it is important to assess whether any changes have occurred in the trap. Another important factor to consider is the degree of penetration of spin traps into the tissue, since not all spin traps are equally effective under physiological pH conditions [44].

The use of real-time quantitative PCR to measure the accumulation of certain transcripts suggests the next potential method for indirectly detecting the short-term existence of $^1\text{O}_2$ [45]. Thanks to extensive research using microchip experiments, RNAseq, as well as Genevestigator databases, it has become possible to accurately identify gene markers that specifically indicate the presence of $^1\text{O}_2$ [46].

Lipid peroxidation is a key downstream effect of ROS and plays a role in cell signaling. The Thiobarbituric Acid (TBA) assay is a common method to measure lipid peroxidation by detecting malondialdehyde (MDA), though it lacks specificity [47]. Advanced techniques like high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) improve specificity by distinguishing between type I (free radical) and type II ($^1\text{O}_2$) lipid peroxidation using hydroxy fatty acids (HOTE) as markers [48].

5 Conclusion

Although the new technologies under development look very promising, both temporal and spatial resolution methods suffer from the weak emission level of $^1\text{O}_2$ luminescence and its rapid attenuation, so quantitative detection of very low concentrations of $^1\text{O}_2$ is currently very challenging, especially with direct optical generation, which is particularly critical for *in vivo* studies.

Chemical probes and traps are characterized by limited penetration into cells or tissues, which will make *in vivo* and *in vitro* studies difficult. It is also worth

considering the environmental conditions in which the probe/trap is located, for example, pH, and the specificity of the substances in question.

As we can see, the described methods have a number of limitations, but a combination of approaches can eliminate some of them. For example, the use of fluorescent probes together with optical luminescence detection can make it possible to determine the total amount of $^1\text{O}_2$ (reacted and returned to the triplet state).

Disclosures

All authors declare that there is no conflict of interest in this paper.

Acknowledgements

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Intermolecular Interaction and Complex Formation of Cytoflavin Components: IR Spectroscopy and Molecular Modeling

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Abstract. IR spectra of intermolecular complexes of the main active components of cytoflavin: succinic acid, nicotinamide and inosine are analyzed. The study focuses on interpreting the absorption band maxima associated with hydrogen bonding between the key functional groups of cytoflavin's constituents. The IR spectra were calculated using density functional theory (DFT) with the B3LYP functional, while experimental IR spectra were recorded using the SpectrumTwo FTIR spectrometer. The results showed that succinic acid has the ability to interact effectively with both one and two nicotinamide molecules by forming stable hydrogen bonds, whereas in complexes with inosine, the strength of the hydrogen bonds is weaker. This suggests that inosine acts as a cofactor to increase the overall stability of the complex.

Keywords: succinic acid; nicotinamide; inosine; cytoflavin; density functional theory; IR spectrum.

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1 Introduction

In medical practice, drugs composed of a combination of active substances are widely used. Each substance exerts its own therapeutic effect and can enhance the overall efficacy compared to individual components. Cytoflavin, a metabolic protease known for restoring antioxidant enzyme activity and exhibiting neuroprotective effects, is commonly employed [1]. Cytoflavin contains succinic acid (10%), inosine (2%), nicotinamide (1%), and riboflavin (0.2%) as active components, with succinic acid being the most significant due to its antioxidant properties. Due to the relatively low proportion of the active substance riboflavin, a coenzyme of glutathione reductase, which restores the pool of the most important component of the antioxidant system of the cell [1], the inclusion of this component in the study of intermolecular interaction with the other active substances of cytoflavin was not carried out. Experimental studies have shown [2] that the positive effects of cytoflavin treatment increase from 60% to 80% when combined with standard therapy methods.

Both nicotinamide and isonicotinamide demonstrate a tendency to form cocrystals with carboxylic acids in three ways [3]. Nicotinamide molecules form cocrystals through strong hydrogen bonds ($N-H\cdots O=C$) (II), while in crystals with succinic acid, strong hydrogen bonds ($N-H\cdots O=C$ and $O-H\cdots O=C$) (III) are observed. In the case of complex formation between succinic acid and nicotinamide, a soft heteromeric acid-pyridine hydrogen bond ($O-H\cdots N$) (I) is formed (Fig. 1).

The aim of this research is to investigate the spectral manifestations of intermolecular interactions between the components of the drug cytoflavin, followed by the determination of the parameters of the resulting hydrogen bonds. This will enable an assessment of the stability of the complex through analysis of the calculated structure and comparison of experimentally measured and calculated IR spectra. The key components of cytoflavin, succinic acid, nicotinamide and inosine were selected as the research objects. Understanding these mechanisms will provide deeper insights into the therapeutic effects of cytoflavin.

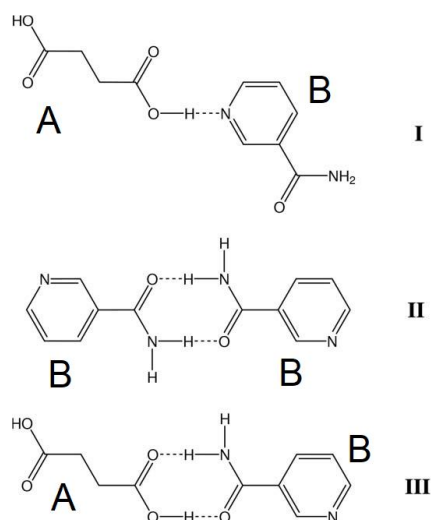


Fig. 1 Potential hydrogen-bond synthons found in acid–amide cocrystals (A – molecule of succinic acid, B – molecule of nicotinamide). (I) Hydrogen bond formed between nicotinamide and succinic acid molecules; (II) Hydrogen bonds formed between two nicotinamide molecules; (III) Hydrogen bonds formed between nicotinamide and succinic acid molecules in the crystals.

2 Methods

The study of intermolecular interactions was conducted by modeling the structures, calculating the normal vibration frequencies, and determining the intensities of IR absorption bands of the molecules and their complexes. These calculations were performed using density functional theory (DFT) [4] with the B3LYP functional [5] and the 6-31G++(d) basis set as this basis set is commonly used in the study of organic molecules [6]. It also includes polarization d-functions for second-row atoms and is supplemented with diffuse functions for heavy atoms and hydrogen [7], which are necessary for the accurate calculation of hydrogen bonding parameters between multiple molecules.

The Chemcraft editor and molecular structure visualizer [8], along with an author-developed program for displaying infrared spectra, were used to recreate the IR spectra based on numerical values obtained from the Gaussian program.

The hydrogen bond energies were calculated using Johansen's empirical formula [9]:

$$-\Delta H = 0.3\sqrt{\Delta\nu - 40}. \quad (1)$$

Finally, the calculated frequencies were multiplied by an anharmonicity correction factor [10–11]. The following scaling factors were chosen: 0.97 for frequencies between 0 and 2000 cm^{-1} and 0.94 for frequencies between 2000 and 4000 cm^{-1} .

Colleagues from RTA Research Center LLC recorded the IR spectra succinic acid and nicotinamide (Figs. 2–3) using a SpectrumTwo FTIR spectrometer (PerkinElmer, USA) equipped with a diffuse reflectance accessory (without tableting in KBr) in the range of 4000–600 cm^{-1} with a resolution of 2 cm^{-1} at a temperature of 20 °C. In the process of preparing materials for recording IR spectra of succinic acid and nicotinamide, suspensions were used on analytical balances with a sampling rate of 0.01 mg of succinic acid 5.6 mg and nicotinamide 5.76 mg. The suspensions were mixed with 200 μl of water, leading to the formation of a poorly soluble precipitate. The precipitate dissolved completely after the addition of 200 μl EtOH and vigorous stirring of the suspension on a vortex. The final crystalline phase of the substances was obtained after drying on a thermo shaker at 100 °C for 2 h. The experimental IR spectrum of inosine was obtained from the open-access IR spectra database – SpectraBase [12].

3 Results

The optimized structures and calculated IR spectra of succinic acid, nicotinamide, inosine and their complexes are presented in Figs. 2–9.

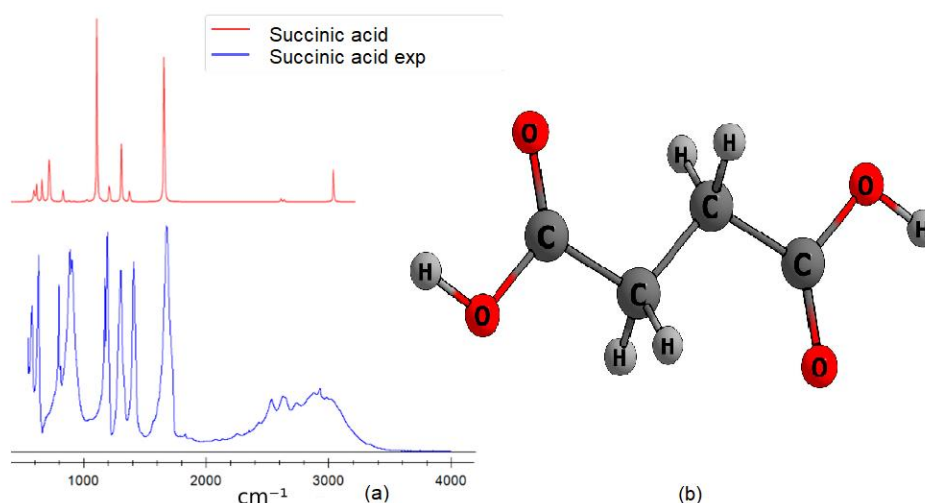


Fig. 2 (a) IR spectrum and (b) calculated structure of succinic acid: $\text{C}_4\text{H}_6\text{O}_4$ (red – calculated, blue – experimental).

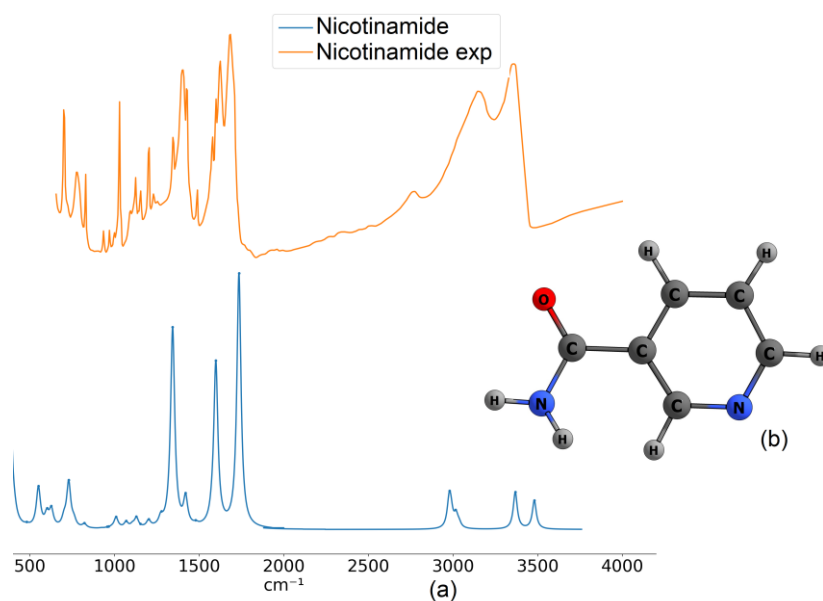


Fig. 3 (a) IR spectrum and (b) calculated structure of nicotinamide: $\text{C}_6\text{H}_6\text{N}_2\text{O}$ (orange – experimental, blue – calculated).

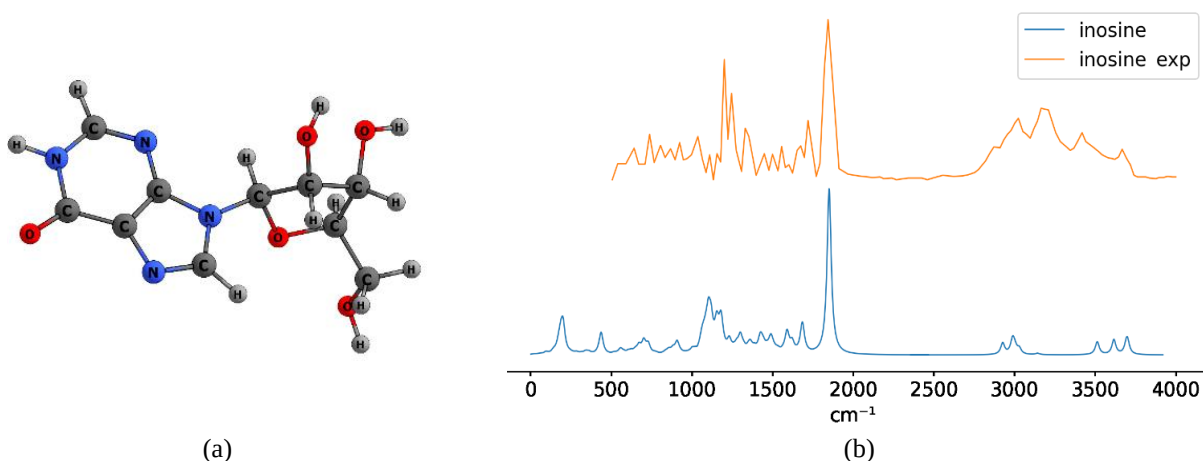


Fig. 4 (a) IR spectrum and (b) calculated structure of inosine: $\text{C}_{10}\text{H}_{12}\text{N}_4\text{O}_5$ (orange – experimental, blue – calculated).

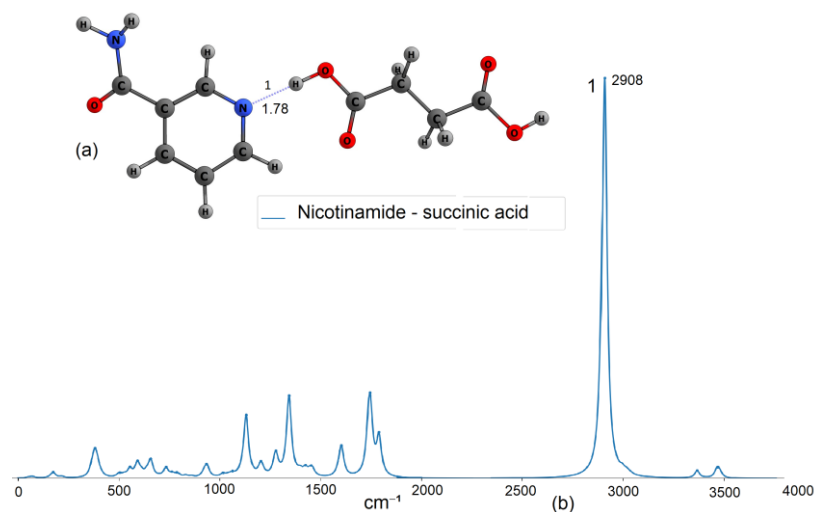


Fig. 5 (a) Calculated structure and (b) IR spectrum of the nicotinamide-succinic acid molecular complex: $\text{C}_4\text{H}_6\text{O}_4 - \text{C}_6\text{H}_6\text{N}_2\text{O}$. The hydrogen bond lengths are marked 1 in Panel (a). The absorption peaks corresponding to -OH (1) group vibrations are marked with number 1 in Panel (b).

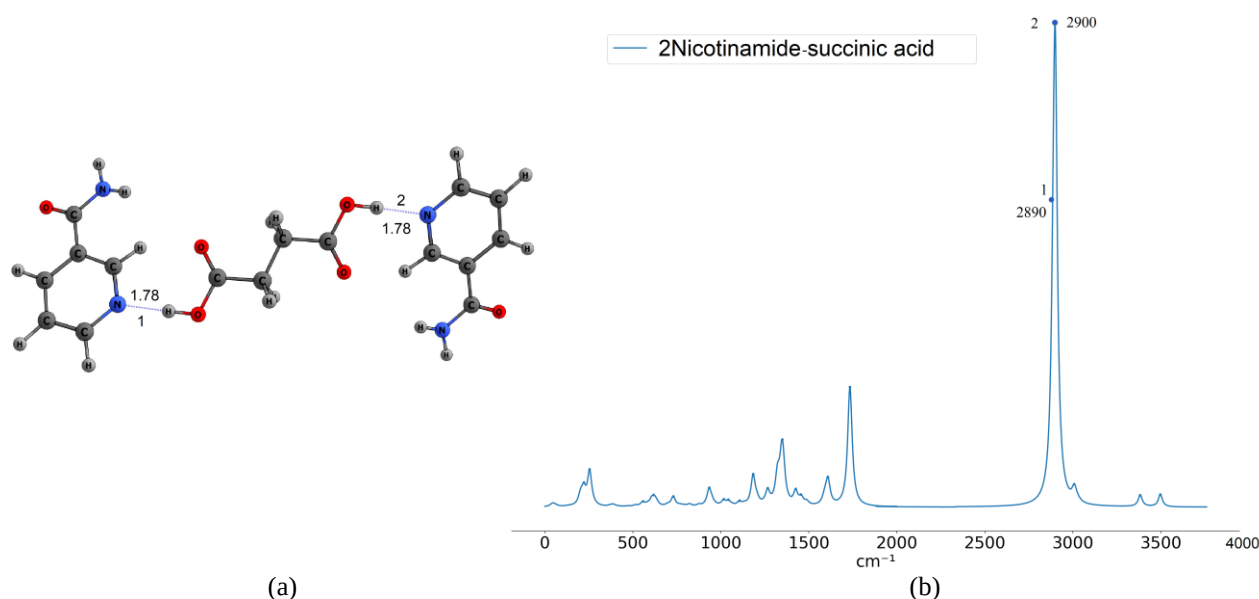


Fig. 6 (a) Calculated structure and (b) IR spectrum of the 2nicotinamide-succinic acid molecular complex: $C_4H_6O_4 - 2(C_6H_6N_2O)$. The hydrogen bond lengths are marked 1 and 2 in Panel (a). The absorption peaks corresponding to -OH (1, 2) group vibrations are marked with numbers 1–2 in Panel (b).

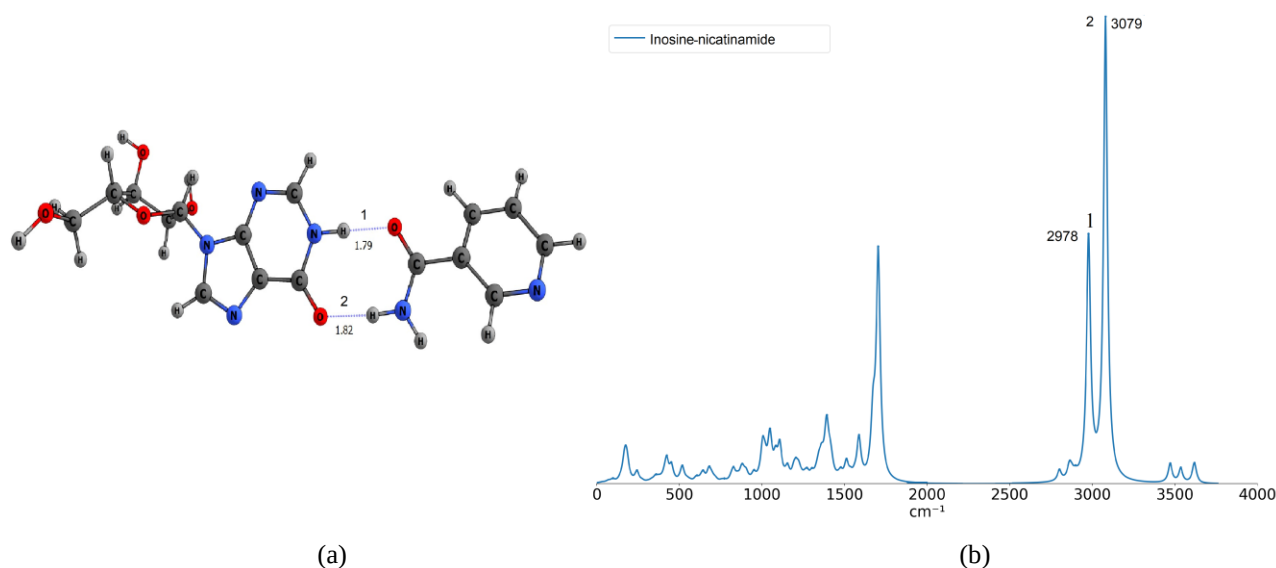


Fig. 7 (a) Calculated structure and (b) IR spectrum of the inosine-nicotinamide molecular complex: $C_{10}H_{12}N_4O_5 - C_6H_6N_2O$. The hydrogen bond lengths are marked 1–2 in Panel (a). The absorption peaks corresponding to -NH (1, 2) group vibrations are marked with numbers 1–2 in Panel (b).

Based on research [13], the structures and IR spectra of the succinic acid and nicotinamide complexes were calculated. The characteristic frequencies of the absorption bands in the calculated complexes correspond to those reported in earlier studies [13]: peaks corresponding to C–H bond vibrations are located in the frequency range of 2860–3090 cm^{-1} , peaks in the 2890–2910 cm^{-1} range correspond to the vibrations of hydrogen bonds in succinic acid and nicotinamide complexes, while the stretching vibrations of free N–H bonds are in the 3300–3500 cm^{-1} range, and free O–H bonds are in the 3400–3500 cm^{-1} range. The intensity and frequency

of the stretching vibrations of the hydrogen bonds in the succinic acid–nicotinamide–inosine complexes are presented in Table 1.

The strength of the formed hydrogen bonds was assessed according to the classification provided in Ref. [14], where strong hydrogen bonds are considered to have energies of 14.34–28.65 kcal/mol and hydrogen bridge lengths of 2.2–2.5 Å. Medium-strength bonds have energies in the range of 3.82–14.43 kcal/mol and hydrogen bridge lengths of 2.5–3.2 Å, while weak bonds have energies of less than 2.87 kcal/mol and hydrogen bridge lengths of 3.2–4.0 Å.

Table 1 Calculated parameters of hydrogen bonds.

Bond number	Bond type	Length of H– Bond <i>R</i> , Å	Length of hydrogen bridge <i>R_b</i> , Å	Frequency <i>ν</i> , cm ^{−1}	Frequency shift <i>Δν</i> , cm ^{−1}	Enthalpy, − <i>ΔH</i> , kcal/mol	Intensity, <i>I_{IR}</i> , km/mol
Nicotinamide-succinic acid, <i>E</i> = −873.97 a.u.							
1	O–H⋯N	1.78	2.79	2908	557	6.82	2232
2Nicotinamide-succinic acid, <i>E</i> = −1290.98 a.u.							
1	O–H⋯N	1.78	2.79	2890	576	6.95	1152
2	O–H⋯N	1.78	2.79	2900	566	6.88	3494
Inosine-nicotinamide, <i>E</i> = −1400.5 a.u.							
1	N–H⋯O	1.79	2.84	2978	389	5.61	1145
2	N–H⋯O	1.82	2.85	3079	288	4.72	2534
Inosine-succinic acid, <i>E</i> = −1440.44 a.u.							
1	O–H⋯O	1.76	2.75	3096	369	5.44	852
2	O–H⋯O	1.84	2.82	3300	165	3.36	773
Inosine-nicotinamide-succinic acid, <i>E</i> = −1857.47 a.u.							
1	N–H⋯O	1.76	2.83	2997	370	5.45	1453
2	O–H⋯O	1.79	2.75	3099	367	5.42	783
3	N–H⋯O	1.85	2.87	3121	246	4.31	2050
4	O–H⋯O	1.83	2.82	3299	167	3.38	760

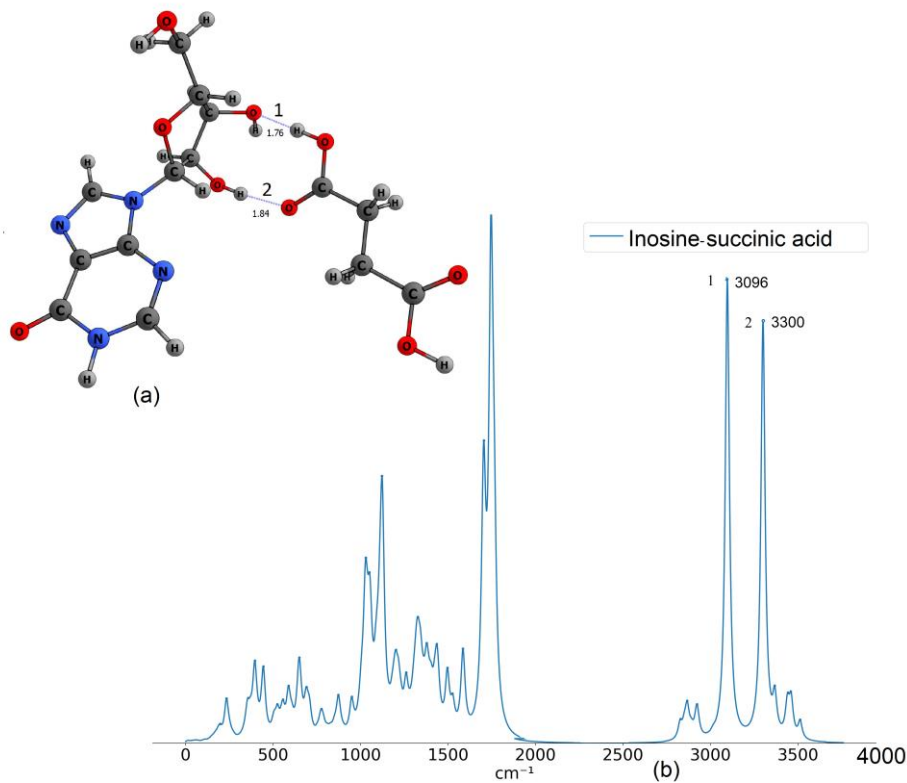


Fig. 8 (a) Calculated structure and (b) IR spectrum of the inosine-succinic acid molecular complex: C₁₀H₁₂N₄O₅ – C₄H₆O₄. The hydrogen bond lengths are marked 1–2 in Panel (a). The absorption peaks corresponding to –OH (1, 2) group vibrations are marked with numbers 1–2 in Panel (b).

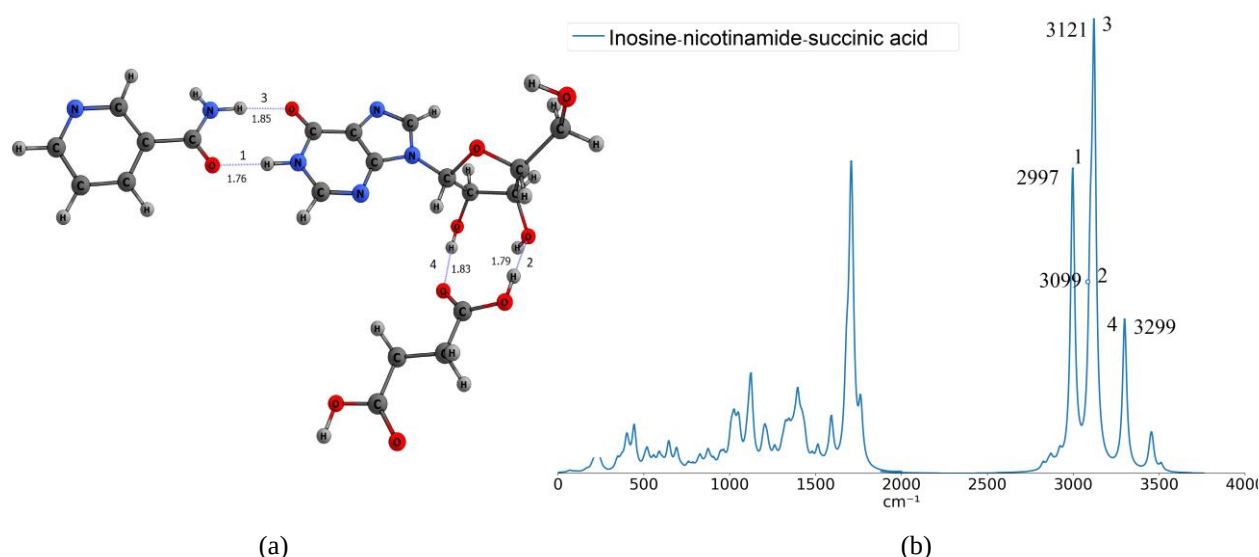


Fig. 9 (a) Calculated structure and (b) IR spectrum of the inosine-nicotinamide-succinic acid molecular complex: $C_{10}H_{12}N_4O_5 - C_6H_6N_2O - C_4H_6O_4$. The hydrogen bond lengths are marked 1–4 in Panel (a). The absorption peaks corresponding to $-OH$ (2, 4) and $-NH$ (1, 3) group vibrations are marked with numbers 1–4 in Panel (b).

The optimization and calculation of the molecular structures of succinic acid and nicotinamide clearly indicate that their interaction ($C_4H_6O_4 - C_6H_6N_2O$) leads to the formation of an $O-H \cdots N$ hydrogen bond. This bond can be characterized as medium-strength, approaching strong, with a frequency shift of 557 cm^{-1} and an intensity of 2232 km/mol .

In the complex formed by two molecules of nicotinamide and one molecule of succinic acid ($C_4H_6O_4 - 2(C_6H_6N_2O)$), two $O-H \cdots N$ bonds are observed. These bonds can be classified as medium-strength, with binding energies of 6.95 and 6.88 kcal/mol , frequency shifts of 576 cm^{-1} and 566 cm^{-1} , and intensities of 1152 and 3494 km/mol , respectively.

When analyzing the experimental and calculated IR spectra of inosine ($C_{10}H_{12}N_4O_5$) (Fig. 4), good agreement was observed in the characteristic absorption band frequencies. Thus, in the frequency range of $2805\text{--}3075\text{ cm}^{-1}$, peaks corresponding to the stretching vibrations of C-H bonds are located. In the interval of $3360\text{--}3370\text{ cm}^{-1}$, stretching vibrations of free N-H bonds are observed, while in the range from 3460 to 3550 cm^{-1} , stretching vibrations of free O-H bonds are present.

In the inosine-nicotinamide complex ($C_{10}H_{12}N_4O_5 - C_6H_6N_2O$), 2 hydrogen bonds of the N-H $\cdots$ O type are formed between the molecules. These bonds can be classified as medium-strength bonds with bond energies of 5.61 and 4.72 kcal/mol , as they exhibit frequency shifts of 389 cm^{-1} and 288 cm^{-1} and intensities of 1145 and 2534 km/mol , respectively.

The calculated structure and IR spectrum of the inosine-succinic acid complex ($C_{10}H_{12}N_4O_5 - C_4H_6O_4$) are shown in Fig. 8. During the complex formation of these molecules, two O-H $\cdots$ O type bonds (1–2) are formed. Bond 1 can be classified as a medium-strength bond, as its bond energy is 5.44 kcal/mol , with a frequency shift of 369 cm^{-1} and an intensity of 852 km/mol . Bond 2 is also classified as a medium-

strength bond, with a bond energy of 3.36 kcal/mol , a frequency shift of 165 cm^{-1} , and an intensity of 773 km/mol .

The calculated structure and IR spectrum of the inosine-nicotinamide-succinic acid complex ($C_{10}H_{12}N_4O_5 - C_6H_6N_2O - C_4H_6O_4$) are shown in Fig. 9. During the formation of the molecular ensemble, two hydrogen bonds of the N-H $\cdots$ O type (1, 3) and two hydrogen bonds of the O-H $\cdots$ O type (2, 4) are formed between these molecules. Bond 1 can be classified as a medium-strength bond, as it has a bond energy of 5.45 kcal/mol , a frequency shift of 370 cm^{-1} , and an intensity of 1453 km/mol . Bond 2 is also a medium-strength bond with a bond energy of 5.42 kcal/mol , a frequency shift of 367 cm^{-1} , and an intensity of 783 km/mol . Bonds 3 and 4 are medium-strength bonds with energies of 4.31 and 3.38 kcal/mol , respectively, as their frequency shifts are 246 cm^{-1} and 167 cm^{-1} , and their intensities are 2050 and 760 km/mol , respectively.

The intensity and frequency of the stretching vibrations of hydrogen bonds between nicotinamide, succinic acid and inosine are presented in Table 1.

4 Conclusions

In the course of a comprehensive study of the components of the drug cytoflavin using quantum-chemical methods, it can be concluded that strong hydrogen bonding contributes to a high degree of complex formation between nicotinamide and succinic acid. Inosine, which is part of cytoflavin, exhibits less pronounced hydrogen bonding and is presumably acting as an auxiliary active substance, promoting the optimization of biochemical processes and enhancing the overall effectiveness of the drug.

Furthermore, supramolecular complex formation is of significant importance, as it enhances the interaction between the components of the drug and contributes to

the stabilization of its structure. This suggests that the combination of succinic acid and nicotinamide plays a key role in the manifestation of the therapeutic effect of cytoflavin.

Disclosures

The authors declare no conflict of interest.

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Photochemical Closed-Loop Ozone-Generating System for Medicine Application

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Abstract. This article introduces a cutting-edge device that could potentially be used to therapy of septic wounds in prospect. The circulation of ozonized air with concentrations of up to 60 ppm provided under a hermetic ozonation application chamber. Subsequently, this air stream is directed to an ozone reactor for re-ozonation. To ensure both the secure fixation of the ozonation chamber to a surface and to prevent ozone leakage it is pressurized below atmospheric pressure, thereby in addition creating a vacuum therapy effect which contributes to the effectiveness of the treatment. Ozone is generated by irradiation of atmospheric air at a wavelength of 172 nm, which eliminates of toxic nitrogen oxides production, as it happens in gas-discharge ozonators. The device has undergone *in vitro* testing for the ozonation of various strains of bacteria and fungi. Leveraging its potential oxidizing capacity, pathogenic culture colonies were entirely killed within a 3-min treatment process. It has also been shown, that under *in vitro* conditions, short-term medium-mediated ozonation inducing reversible oxidative stress has a positive effect on proliferative activity and adhesion ability in eukaryotic mesenchymal cells. Depending on ozone concentration, duration and exposure conditions, the diverse effects of ozone, establish a foundation for potential therapeutic applications in the treatment of various diseases.

Keywords: ozone; photochemical ozonator; VUV radiation; antibacterial effect; eukaryotic cells.

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1 Introduction

The advancement of non-medicinal treatment methods and the reduction of antibiotic usage are pivotal components of contemporary medicine. Overreliance on antibiotics has led to the emergence of antibiotic-resistant bacteria, posing a significant threat to human health. Non-medicinal approaches provide compelling alternatives to antibiotics. Ozone, in particular, acts as a potent oxidizing agent, showcasing a notable capacity to readily react with organic substances. This distinctive property positions ozone as a highly effective disinfectant. Unlike antibiotics and ultraviolet radiation, ozone functions through a non-specific mechanism, circumventing the development of habituation (resistance) in microorganisms. The process of ozonation

leads to the oxidation and destruction of bacterial or fungal cell membranes without inflicting damage to their DNA – a notable departure from the effects commonly associated with antibiotics or UV radiation.

Scientific evidence [1] supports various properties of ozone, including its effectiveness as a disinfectant, oxygen donor, immunomodulator, inducer of antioxidant enzymes, metabolic enhancer, and inducer of endothelial nitric oxide synthase. However, it is crucial to acknowledge that ozone, despite its beneficial properties, has inherent toxicity and poses respiratory hazardous risks. When employed invasively, it is imperative to exercise caution and adhere to recommended concentrations and doses.

Ozonation therapy, involving the direct application of ozone to a wound, has been utilized for several decades

to treat septic wounds in both humans [1] and animals [2]. This method has shown particular effectiveness in addressing challenging wounds with diverse pathologies. To administer ozone therapy, a special plastic bag is usually placed over the patient's extremity (arm or leg), and ozonized air is injected into the enclosed space [3]. Following wound ozonation, excess pressure is released through a separate tube, ensuring utilization of ozone.

A local ozone therapy (ozone treatment over a wound) resolves the problem of the regional hypoxia in a purulent wound by activating succinate-dehydrogenase and cytochrome c oxidase. The metabolic recovery of tissue is accompanied by elimination of acidosis, improvement of the microcirculation and nourishment of the inflammatory tissues, acceleration of wound healing processes and epithelialization. [4]. The therapy also increases the susceptibility of microbes to antibiotics, which allows for the reduction of the dose and duration of their administration. The wound surface undergoes rapid bacterial decontamination, necrolysis, granulation, and epithelialization along the edges of the wound. In the conditions of ozone therapy, the pain syndrome also disappears after 3–4 days [5].

When pumping ozonized air or oxygen through the bag under pressure, leaks of ozone that are dangerous for breathing are almost inevitable, so surgeons are reluctant to use this method in hospital settings [1]. To apply this method, it is necessary to recycle the used ozone in an O_3 reactivator or install a powerful ventilation system.

Beyond ozone therapy, a vacuum-assisted closure, also known as negative pressure wound therapy, is used for non-healing or at risk of non-healing wounds. It involves the application of a vacuum dressing over the wound, creating negative pressure [6, 7], which causes microscopic deformation of the wound surface, inducing a gentle stretching effect that encourages division and proliferation of cells. Furthermore, it stimulates tissue growth and angiogenesis [8], while removing enema fluid and exudate from the extracellular space. Additionally, vacuum therapy aids in the elimination of inflammatory mediators and cytokines [6].

Despite the fact that the treatment of septic wounds by ozonation has been carried out for several decades [1] using commercially available medical ozonizers, such as those produced by LEPSE (Russia) or Häsler Medical GmbH (Germany), they nevertheless have some disadvantages. The ozonation system described in the paper, on the contrary, allows eliminating these shortcomings and using ozone therapy in medical institutions more effectively and safely.

2 Methods and Materials

2.1 Photochemical Ozone Generation

Industrial ozonators based on gas discharge into the air were developed in the mid-19th century. To achieve high concentrations of ozone, oxygen is passed through an ozonator. The introduction of photochemical ozonators is

linked to the advancement of potent sources of ultraviolet radiation with a wavelength of less than 200 nm (Vacuum Ultra Violet (VUV) range) [9]. Radiation in this range is characterized by the strong absorption of oxygen, and the energy of quantum is sufficient for the decomposition of O_2 and the formation of O_3 (Fig. 1). Consequently, VUV radiation and three oxygen molecules make two ozone molecules. Similar processes occur in the upper layers of the Earth's atmosphere during the formation of the ozone layer.

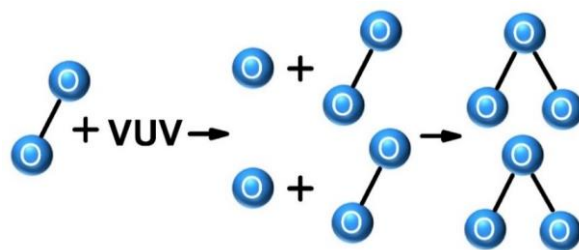


Fig. 1 Photochemical reaction of ozone formation.

The main advantage of photochemical ozonators is as follows: since inert gases (including nitrogen) are transparent in the VUV range, at irradiating the air the radiation directly affects oxygen, which absorbs all VUV radiation in a layer of no more than 5 mm. This means that all the VUV light energy produced by the emitter is utilized for ozone formation. Thus, within the air flow, using a relatively compact device, it is possible to achieve an ozone concentration up to 60 ppm without the need for pure oxygen.

As it is known nitrogen oxides are formed under conditions of high temperature (more than 1000 °C) or as a result of plasma-chemical reaction. There is a necessity to use pure oxygen in gas-discharge ozonators for medical applications, because of NO_x or N_yO_x producing in the interelectrode space in atmospheric air [10], which is toxic to living systems, especially to fresh epithelium during wound healing. Production of nitrogen oxides is excluded in photochemical ozonators by reason of ozone formation driven by VUV (not as a result of plasma-chemical reaction in air).

In the beginning a compact photochemical ozone reactor in the shape of a cylindrical metal tube measuring $\varnothing 40 \times 200$ mm was developed. In this tube, a xenon excimer Dielectric Barrier Discharge (DBD) lamp is coaxially positioned. There is an axial-flow fan at the tube's terminus. The ozone reactor has hermetically fixed fittings at both ends for connection of flexible hoses to pump the air.

The xenon DBD excimer lamp, measuring $\varnothing 21 \times 170$ mm, is equipped with an inner electrode made of aluminium foil, applied a high pulse voltage, while the outer grid electrode is grounded (Fig. 2). The radiation power of the excimer lamp in the VUV range was 1 W at a power consumption of 32 W and plus frequency of 60 kHz. The efficiency of the power supply & lamp system was therefore 3.1%. A VUV power density was measured by UV power meter Hamamatsu C9536

(Shizuoka, Japan) with head H9535-172 on the lamp surface. With the value of the emitting area of the lamp, the total VUV power was calculated. The design and power supply of excimer DBD lamps are comprehensively described in Refs. [11, 12]. Fig. 3 illustrates the emission spectrum of the xenon lamp, as measured by the Ocean Optics Maya 2000Pro spectrometer (Orlando, USA).



Fig. 2 A xenon DBD excimer lamp.

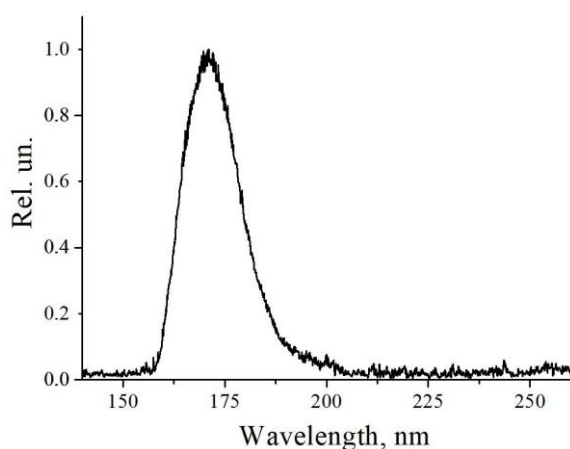


Fig. 3 Emission spectrum of Xe_2^* molecules.

The designed photochemical ozonator has the capability to generate an ozone flow with a concentration reaching up to 60 ppm at an air pumping rate of 8 l/min and VUV radiation power of 1 W. The value of O_3 concentration in the closed system was mainly determined by the radiation power of the DBD excimer lamp and a lesser degree by the flow rate of ozonised air, therefore, in order to ensure better cooling of the lamp, the air pumping rate was 8 l/min. Measurements of ozone concentration were carried out using the ENVEA UV Ozone Analyzer O342e (Poissy, France).

A notable advantage of the developed photochemical ozonator is ability to precisely adjust ozone concentration by linearly adjusting the VUV radiation intensity of the excimer lamp. It was achieved by a special mode of lamp excitation in which pulses of $\sim 2 \mu\text{s}$ duration with relatively long pauses between them are applied to the electrodes [13]. If the excitation energy of a pulse does not change, the excitation power and the VUV power can be linearly adjusted by the pulse frequency. In this way, a linear adjustment of ozone concentration from 5 to 60 ppm was realised. In addition, a relatively low supply voltage of the ozonator enhances compatibility with other electronic medical equipment.

2.2 Closed-Loop Ozone-Generating System

As was already mentioned, to ozonate a wound on a patient's limb (arm or leg), a plastic bag is used, and ozone is pumped into it. However, it is quite challenging to achieve a leak-proofness when the wound is on plane parts of the body (for instance, the abdomen, thigh, back, etc.). To address this issue, an ozonation vacuum chamber **1** has been developed (Fig. 4); it has flexible hoses (**2** and **3**) connected to it for the supply and withdrawal of ozonized air to the ozone reactor **4**.

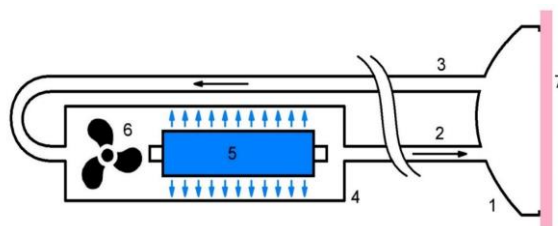


Fig. 4 The photochemical ozonation system: **1** – application chamber, **2** – ozone supply channel, **3** – ozone withdrawal channel, **4** – ozone reactor, **5** – VUV lamp, **6** – fan, **7** – treated surface.

By securely fixing application chamber **1** onto treated area **7**, a leak-proofness is achieved due to the close-loop of ozonation system via channels **2** and **3**. Activation of the fan **6** and lamp **5** initiates a cyclical airflow along the close-loop. In each cycle through the reactor **4**, the air undergoes ozone enrichment, and then it traverses through the chamber **1**, contributing to the treatment of the patient's wound. To securely affix the chamber **1** to the wound, a controlled sub-atmospheric negative pressure ranging from 675 to 710 mm Hg (-75 to -40 mm Hg relative to atmospheric pressure) is maintained in the ozone pumping close-loop. This is achieved by integrating a hermetic outlet into an ozone reactor **4** (not depicted in Fig. 4), and connecting it to a vacuum pump. A microprocessor control unit, along with a pressure sensor integrated into the reactor, monitored the pressure within the closed-loop ozone-generating system. This control unit facilitates the adjustment of ozone concentration levels and monitors the duration of the ozone therapy procedure.

The close-loop of ozonation system, coupled with the negative pressure maintained, effectively eliminates any potential ozone leaks, ensuring the safety of ozone therapy for both the medical personnel and the patient. Additionally, the versatility of the system allows for adaptability in the type of the application chamber **1**. For instance, it can be substituted with a plastic bag for treating limbs or replaced with an overlay chamber of varying dimensions suitable for different wound contours and the relief of different body areas. This flexibility may enhance the applicability of the ozonation system in perspective, catering to a range of therapeutic requirements with precision and convenience. Through the creation of a low-level vacuum within the chamber **1**,

the system also may contribute to the expeditious healing of septic wounds.

Notably, the system's control unit features a vacuum therapy function without wound ozonation. In this mode, both the fan 6 and excimer lamp 5 remain inactive, and the control unit establishes negative pressure within the application chamber based on the parameters set by the medical professional.

3 Results and Tests

3.1 General Design of the Closed-Loop Ozone-Generating System

The system is a prototype of medical device designed to administer ozone treatment to surfaces beneath the application chamber or within a plastic bag. The functional diagram of the system is given in Fig. 5. The main part of the control unit is an operation panel 1 featuring a built-in microprocessor and a touch screen. The operation panel 1 connected with a 4-channel relay module 2 and a pressure sensor 7. This configuration makes it possible to independently activate four components of the system as needed: power supply 3 of VUV excimer lamp, fan 4, fill-up valve 5 and vacuum pump 6. The outlet of the vacuum pump was connected to a carbon-based ozone destructor (not shown on the Fig. 5). The operation panel 1 can manage and regulate the pressure within the ozone reactor through the integrated pressure sensor 7 and vacuum pump 6. VUV lamp excitation timing pulses were generated from the control panel 1 and applied to the power supply 3 at a frequency corresponding to the set ozone concentration level.

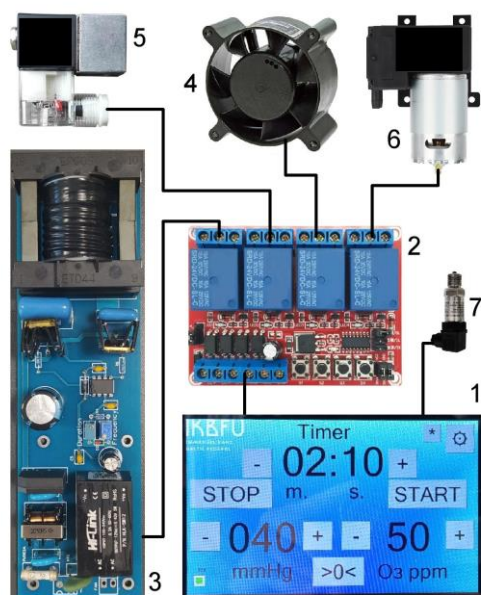


Fig. 5 Functional diagram of the system: 1 – operation panel, 2 – relay module, 3 – power supply, 4 – fan, 5 – fill-up valve, 6 – vacuum pump, 7 – pressure sensor.



Fig. 6 Closed-loop ozone-generating system.

All parts of the system are arranged within in a case measuring $325 \times 220 \times 120$ mm (Fig. 6). Two fittings, located on the back panel of the case, are used to attach flexible hoses for the cyclical pumping of ozonized air through the chamber or bag, each hose measuring a length of 70 cm.

Prior to commencing ozonation, it is essential to set the required ozone concentration, procedure duration, and the negative pressure level on the operation panel screen. Once the treated area is appropriately covered with the chamber, pressing the “START” button sets off the vacuum pump, evacuating air from the ozone reactor up to the set pressure level. Following this, the VUV lamp automatically switches on, the fan 4 (Fig. 5) starts to operate, and the countdown for the procedure time commences.

If the application chamber is not properly contacted with treated surface thus preventing the air from being pumped out of the ozone reactor, a related message appears on the operation panel screen. If, for any reason, the pressure in the chamber exceeds the set value, the vacuum pump is activated to achieve the required pressure parameters. Pressing the “STOP” button pauses the procedure, and pressing the “START” button resumes it. Upon the expiration of the set time or by pressing “STOP” twice during the procedure, the system operation is ceased, and valve 5 (Fig. 5) opens to allow air into the ozone reactor. At the moment the vacuum pump 6 was switched on for 90 s to remove the residual ozone from the entire system volume.

3.2 Evaluation of the Antibacterial Effect of Ozone

The pathogenic strains selected for the study included *Escherichia coli* ATCC 25922 (Gram-negative conditionally pathogenic bacterium), *Bacillus subtilis* (a gram-positive, spore-forming, facultative aerobic bacterium), *Candida albicans* EMTC 34 (the causative agent of opportunistic infections), and *Aspergillus niger*

(a mold fungus capable of inducing mucormycosis in humans).

The mechanisms of action and metabolic processes of the selected bacteria are identical to other Gram-negative and Gram-positive bacteria, and some of them are antibiotic resistant, making them a standard bacterium present in wounds [14, 15] so they were taken as a case study to investigate wound infections.

C. albicans – yeast-like microscopic fungi, which can also be used to assess the effectiveness of antifungal therapy for wounds. *A. niger* is capable of causing disseminated invasive aspergillosis, an infection that spreads widely throughout the body, including on the wound surface [16]. These fungi also have similar metabolism to other microscopic fungi, and can be used as example microorganisms to investigate the efficacy of wound infection treatment [16].

All test strains were inoculated into Petri dishes with agarized medium and cultured following the protocol described in Ref. [17]. Subsequently, the Petri dishes were placed on a rubber mat and securely sealed with the application chamber of the ozonator. The ozone exposure duration at a concentration of 60 ppm was 3 min. Following ozonation, the test strains were incubated on agarized growth medium at 37 °C (*A. niger* at 28 °C) for 24–48 h. Petri dishes in which the same microorganisms were cultured were used as a negative control; however, their suspensions were not treated with ozone. The test strains in the control Petri dishes were co-incubated with the ozone-treated Petri dishes.

The experiment was conducted in two repetitions. The presence of an antibacterial effect of ozone

(sensitivity of microorganisms to the action of ozone) was evaluated by counting colony-forming units (CFU/ml) on inoculated media *in vitro*. In cases of no bacterial growth, a value of <10 CFU/ml (detection limit) was used [18–20]. Some of the results are shown in Table 1 and Fig. 7. Notably, gaseous ozone showed a strong antimicrobial effect, leading to the complete eradication of all microorganisms examined. This included both Gram-negative and Gram-positive bacteria, as well as yeast-like and mold fungi. It demonstrates the effectiveness of gaseous ozone in eliminating a wide range of pathogens. Analysis of the data presented in Fig. 7 also indicates that ozone treatment resulted in complete prevention of the growth of opportunistic microorganisms.

Table 1 Ozone treatment of *E. coli*, *B. subtilis*, *C. albicans* and *A. niger*.

Microorganism	Without ozone treatment, CFU/ml	After ozone treatment, CFU/ml
<i>E. coli</i>	$6.3 \cdot 10^6 - 10^7$	<10
<i>B. subtilis</i>	$3.2 \cdot 10^7 - 10^8$	<10
<i>C. albicans</i>	748.2	<10
<i>A. niger</i>	976.6	<10

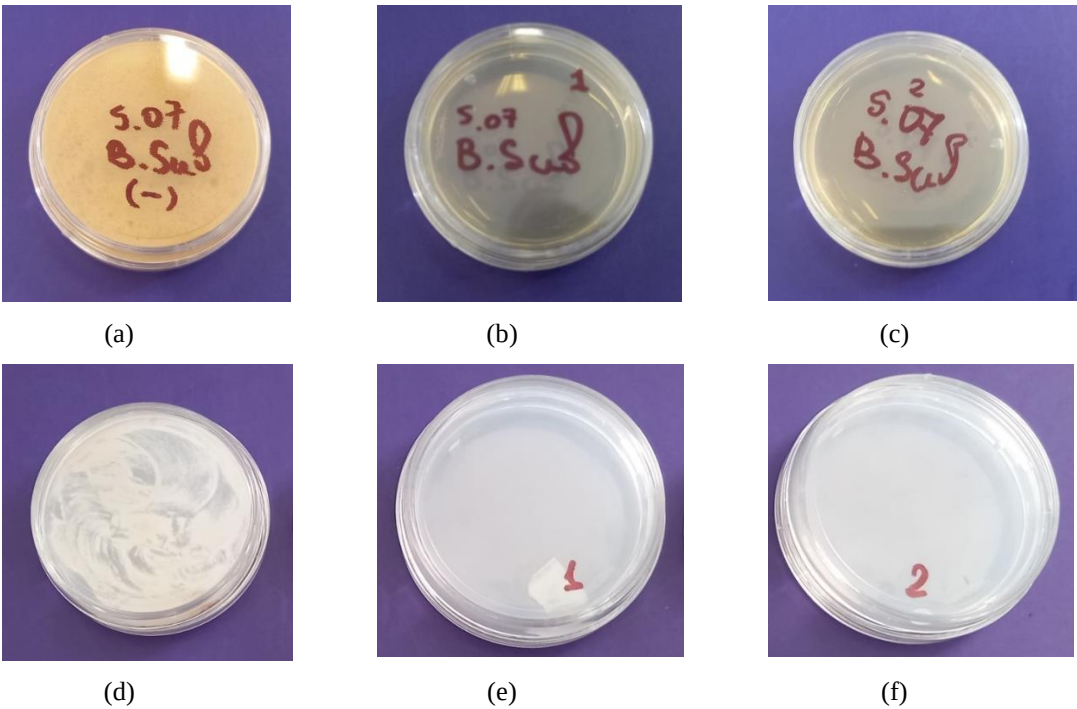


Fig. 7 (a–c) *B. subtilis* and (d–f) *A. niger* after incubation: (a, d) control (no ozone exposure), (b, e) after ozone treatment (repetition 1), (c, f) after ozone treatment (repetition 2).

The bactericidal effect observed in the studies corroborates the previously established data on the non-specific antibacterial efficacy of ozone against prokaryotic organisms [15–16]. High doses of ozone cause bacterial cell death by disrupting the lipids and lipoproteins of cell membranes and triggering cell death pathways (apoptosis). Additionally, ozonation disrupts bacterial populations by affecting adhesion proteins and mechanisms involved in the formation of intercellular matrix and intercellular bonds within bacterial biofilms, supporting the notion that high-dose ozonation is an effective non-specific disinfection strategy.

Other studies have also demonstrated that short-term exposure to high ozone concentrations leads to the activation of a series of anti-inflammatory and regenerative mechanisms in the cells and tissues of humans or animals, activating signalling pathways and subsequent synthesis of a complex of anti-inflammatory, regulatory, and growth factors [20–21].

3.3 The Effect of Ozonation on Eukaryotic Cells

Human multipotent mesenchymal stromal cells (MMSCs) of adipose tissue have been isolated by enzymatic method from the lipoaspirates of healthy donors obtained during the esthetic liposuction. The lipoaspirates were provided for research purposes by partners of the plastic surgery clinic. The receipt and transfer of the material was carried out in accordance with the ethical and legal norms in force on the territory of the Russian Federation, with the signing of informed consent, in accordance with the procedure approved by the Local Ethical Committee of the Innovation Park of the Immanuel Kant Baltic Federal University (permission No. 7 on 9 December 2015). Multipotent stem cells have been obtained from the lipoaspirates and cryopreserved using liquid nitrogen in the cryobank of the Center for Immunology and Cellular Biotechnology of the Immanuel Kant Baltic Federal University. For our study,

MMSCs from the cryopreservation have been morphologically stabilized on plastic, cleaned up of debris by a cascade of washing procedures and removed from plastic by trypsinization and introduced into model cultures on a 6-well plate after achieving confluency of 80%.

Experiments were conducted on healthy stem cells primary cultures, since these cells are among the first cells recruited in the remodeling of the tissues of a wound. The fifth passage of human MMSCs derived from subcutaneous adipose tissue were used for an *in vitro* experiments. Cells were expanded on plastic under standard conditions [22]. Cells ozonation contact was under both during direct and indirect (mediated a liquid medium) conditions, the exposure time was 3 min.

The experimental cultures were analysed using an inverted microscope with an integrated OLYMPUS Video Zavr Standard camera (Tokyo, Japan) for the morphological characteristics, INVITROGEN Countess™ Automated Cell Counter (Waltham, USA) with Trypan blue 0.4% was used to determine the viability parameters. The relative number of apoptotic cells was assessed by flow laser cytometry using a MILTENYI BIOTEC MACS Quant Analyzer flow cytometer (Bergisch Gladbach, Germany) following the manufacturer's protocols.

The experiments showed that the morphology of eukaryotic cells of vertebrates did not change during medium-mediated contact with ozone (Fig. 8). However, direct ozonation led to various changes in cell morphology. The degree of such morphological changes associated with severity of the exposure conditions.

The number of live cells was increased by 22% after 24 h while exposed to ozone for 3 min (model 2E – ozonation of a medium, followed by the 3-min ozonation of cells in a monolayer on plastic, after which the medium was removed) (Fig. 9). In the control cultures (1K – 3K are control cultures undergoing the same experimental cycle, but without ozonation) this indicator was 31%, under the same conditions.

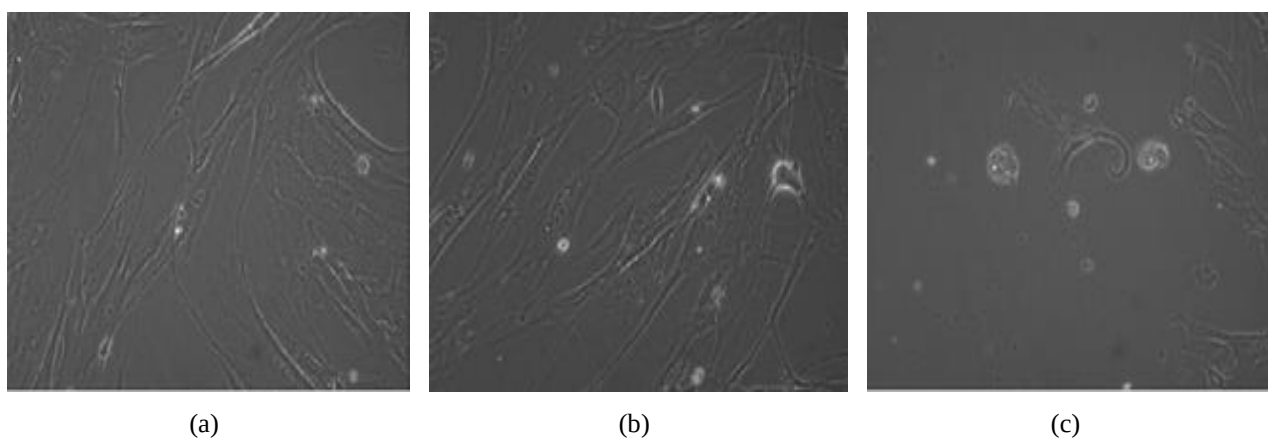


Fig. 8 Morphological changes of MMSCs in different ozonation models (magnification $\times 200$). (a) Control cells (without ozonation), (b) after medium-mediated ozonation (3-min exposure), (c) cells after short-term direct ozonation (3-min exposure).

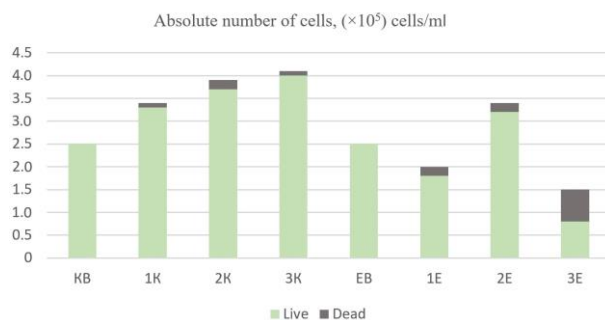


Fig. 9 Absolute indicators of culture viability in the control and experimental models of MMSCs: KB in the control models before the experiment; 1K – 3K in the control models after 24 h; EB in experimental models before the experiment; 1E – 3E in experimental models after 24 h.

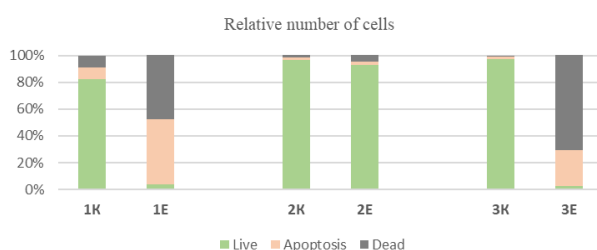


Fig. 10. Relative number of live, dead, and apoptotic MMSCs obtained by flow cytometry: 1K – 3K in the control models after 24 h; 1E – 3E in the experimental models after 24 h.

Using these models, the highest survival ratios of the culture could also be demonstrated. Both direct exposure to ozone (model 1E cells were ozonated without the presence of nutrient medium only in a humid atmosphere), and indirect exposure with prolonged cultivation in ozonated medium (model 3E – the ozonated medium surrounding the cells at the time of exposure was not changed, and the cells were for left for 24 h in a state of hyperoxygenation) led to a decrease in the absolute number of live cells in the culture (1.5–2 times) (Fig. 10).

4 Conclusion

The prototype closed-loop ozone-generating system, unlike the O₃ generators used for ozone therapy, has a special mode of operation in which ozone is supplied to the application chamber and then returned for re-ozonisation. To avoid O₃ leakage, ozonised air with a concentration of up to 60 ppm is cyclically pumped through a sealed, closed-loop ozone-generating system under negative pressure. The ozone-generating closed-loop system is primarily designed for the safe and effective treatment of septic wounds with high concentration ozone without the need for an oxygen supply, and has the ability to perform vacuum therapy.

The ozone flow emanating from the system exhibits potent antibacterial and fungicidal properties. Leveraging its potential oxidizing capacity, pathogenic culture colonies were entirely killed within a 3-min treatment process. This suggests that the suggested ozone treatment method holds considerable potential for diverse applications in both the medicine and food industries.

Our study also showed that *in vitro* conditions, short-term medium-mediated ozonation inducing reversible oxidative stress has a positive effect on proliferative activity and adhesion ability in eukaryotic mesenchymal cells.

Depending on ozone concentration, duration and exposure conditions, the diverse effects of ozone, establish a foundation for potential therapeutic applications in the treatment of various diseases. This includes leveraging cytotoxic/cytostatic effects in oncology and aiming to stimulate reparative regeneration processes in cases of chronic inflammatory conditions and non-healing wounds, even those complicated by antibiotic-resistant bacterial microflora.

Disclosures

The authors have no relevant financial interests in this article and no potential conflicts of interest to disclose.

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Analysis of Betulin Complex Formation with Polar Solvents Using DFT Methods

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Abstract. The efficiency of betulin extraction from plant material is highly dependent on the choice of solvent, particularly due to the role of hydrogen bonding in determining solvation behavior. This study aims to investigate the molecular interactions between betulin and polar solvents, focusing on hydrogen bond formation, to elucidate the mechanisms underlying solvent efficiency in betulin extraction. Using density functional theory calculations with 6-31++G(2d,2p) basis and IR spectral analysis, the interactions between betulin and polar solvents (acetonitrile, isopropanol, acetone, and ethanol) were examined. The results revealed that ethanol forms the strongest and most stable hydrogen bonds with betulin, which correlates with its experimentally observed superior extraction efficiency. This work underscores the importance of hydrogen bonding in solvation behavior and offers valuable insights for the development of efficient extraction protocols in natural product chemistry. Additionally, understanding betulin's molecular interactions with solvents can open new possibilities for its applications in biophotonics, where its unique structural and optical properties may be utilized.

Keywords: betulin; density functional theory; infrared spectra; polar solvents; extraction.

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1 Introduction

Betulin, a pentacyclic triterpenoid from the lupane group (Fig. 1), is widely distributed in nature, particularly in birch bark. Lupane series triterpenoids possess a complex polycyclic framework built upon a sterane core, which integrates four cyclohexane rings and a fully saturated cyclopentane ring. A key structural characteristic of betulin within this class is the inclusion of a five-membered ring along with an α -isopropenyl substituent [1]. Due to its potential biological properties, including antitumor and antiviral activity [2–4], this compound has generated interest in the fields of pharmacology and natural product chemistry.

Triterpenoids are crucial in enhancing plant defense systems against infections from a variety of pathogens. Their antiviral properties are commonly linked to hindering the initial stages of viral attachment and entry into host cells. This interference subsequently

disrupts viral replication process once infection has occurred [5–6].

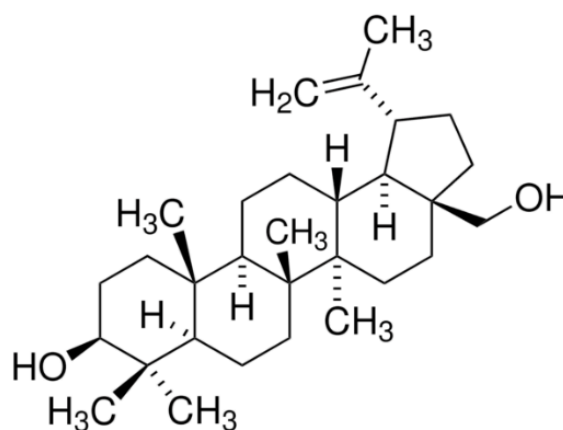


Fig. 1 The structural formula of betulin.

While betulin therapeutic potential has been extensively explored, an emerging area of interest is its possible role in biophotonics. Betulin's unique chemical structure and biological effects suggest it could contribute to this field, particularly in enhancing light-mediated diagnostic and therapeutic approaches. Betulin's pentacyclic triterpenoid structure provides a robust platform for chemical modifications, enabling creation of derivatives with enhanced solubility, stability, and functionality. Key derivatives include betulinic acid, betulin esters, betulin-based polymers, and hybrid materials such as betulin-nanoparticle conjugates [7–8]. These modifications expand the applicability of betulin in biophotonic applications.

Betulinic acid, a derivative of betulin, can be conjugated with fluorescent dyes to create probes for bioimaging. These probes are useful in techniques such as fluorescence microscopy and optical coherence tomography (OCT), enabling high-resolution imaging of cellular and tissue structures. For example, betulinic acid-based probes have been used to track cancer cells *in vitro* and *in vivo*, providing valuable insights into tumor biology [9].

Betulinic acid and its esters have been explored as photosensitizers in photodynamic therapy. Upon light activation, these compounds generate reactive oxygen species (ROS), which can selectively destroy cancer cells. This approach minimizes damage to healthy tissues and offers a promising alternative to conventional cancer treatments [10–11].

Betulin esters and polymers can be incorporated into nanoparticles or hydrogels for light-triggered drug delivery. For instance, betulin-based hydrogels have been developed to release therapeutic agents in response to specific wavelengths of light, enabling precise control over drug release kinetics. This technology is particularly useful for targeted cancer therapy and wound healing [12–13].

The extraction of betulin from plant material is a critical process for obtaining this valuable compound in its pure form. This purified betulin can then be used in a variety of industries, including the pharmaceutical industry, where it serves as a precursor for the synthesis of betulinic acid, another compound with significant medicinal properties. To ensure the success of the extraction process, it is important to carefully select a solvent that is compatible with the solvation interactions and polarity of the material being extracted. The choice of solvent has a significant impact on the efficiency of the extraction [14], as it determines not only the yield but also the purity and stability of the extracted compound. Factors such as solvent polarity, temperature, and extraction time must be optimized to achieve the best results.

In a study conducted by Levdansky et al. [15], the extraction of betulin from birch bark using various organic solvents with different polarities was investigated. The results demonstrated that the use of polar solvents allows for the production of extracts with yields up to 28.8% and betulin contents ranging from

90% to 95%. (Fig. 2). Researchers have noted that as the temperature and polarity of the solvent increase, the total volume of the extract increases, but the percentage of betulin in the extract decreases [15, 16]. While these experimental findings provide valuable insights into the extraction process, the molecular-level mechanisms governing the interactions between betulin and solvents remain unclear. To address this gap, density functional theory (DFT) calculations can be employed to investigate the nature and strength of these interactions.

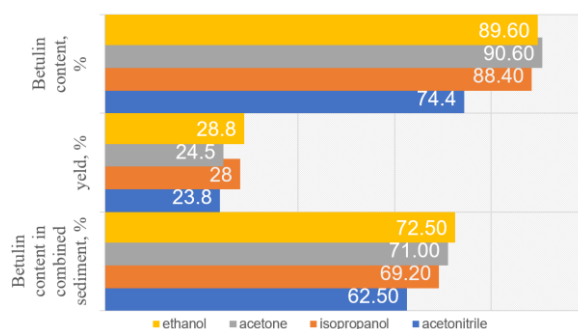


Fig. 2 Content of betulin in the extract, yield, and betulin content in the combined precipitate depending on the solvent, %. The data was taken by Levdansky et al. [15].

Hydrogen bonds represent a critical class of intermolecular forces, exerting substantial influence on the structural and functional characteristics of biological molecules. These bonds may be especially significant in the context of betulin, as they can influence its behavior in different chemical and biological settings. For instance, hydrogen bonding can affect the solubility, stability, and reactivity of betulin, which in turn impacts its extraction efficiency and subsequent applications.

This study aims to employ advanced computational methods, particularly DFT, to simulate and analyze the molecular interactions between betulin and various polar solvents. By systematically examining hydrogen bond formation, binding energy, and vibrational spectral changes, we seek to elucidate the molecular mechanisms governing betulin solvation. Understanding these interactions is crucial for improving solvent selection in betulin extraction, thereby enhancing both yield and purity.

2 Methods

To theoretically substantiate the extraction ability of various polar solvents, molecular modeling was performed, and IR spectra were calculated using the DFT method [17] in Orca [18]. B3LYP functional [19, 20] and 6-31++G(2d, 2p) basis set [20] were used for the calculations. The DFT calculations provided detailed insights into the energetics and geometries of hydrogen bond formation, while IR spectroscopy validated the theoretical findings by identifying

characteristic vibrational modes associated with betulin – solvent interactions.

Since intermolecular interaction is based on the formation of hydrogen bonds through the hydroxyl groups of betulin, there are only two potential possibilities for the attachment of a polar solvent, which we will consider (Fig. 3). Explicit solvation was performed, but to increase the accuracy of calculations, we also used implicit solvation of water using the conductor-like polarizable continuum model (CPCM) [21] method.

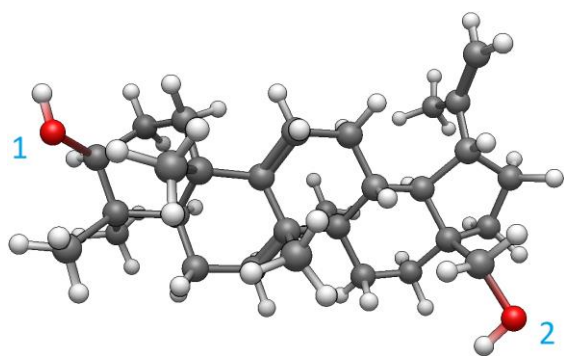


Fig. 3 Betulin structure. 1, 2 – sites for the attachment of a polar solvent.

The Avogadro editor – visualizer of molecular structures [22] and the author's program for displaying infrared spectra, which allows recreating the IR spectrum based on numerical values obtained by Orca program, were used for the work.

To calculate the energy of hydrogen bonds between betulin and solvents, methods related to computation of the isolated fragments energies and Johansen's empirical formula will be employed. The method energies of isolated fragments (Eq. (1)) will be calculated to determine the interaction energy, while the Johansen formula [23] will be applied to further analyze and validate the results. Isolated fragment approach is based on computational analysis of the betulin-solvent complex (Eq. (1)). Simultaneously, the approach based on Johansen's formula (Eq. (2)) employs an empirical formula, which is commonly used by experimental researchers, with primary focus placed on determining the bond energy through analysis of frequency shifts.

$$E_{\text{interaction}} = E_{\text{complex}} - (E_{\text{betulin}} + E_{\text{solvents}}), \quad (1)$$

$$-\Delta H = 0.3\sqrt{\Delta\nu - 40}. \quad (2)$$

For the evaluation of hydrogen bonds, the classification presented in Table 1 of reference [24] will be utilized. This classification provides a systematic framework for analyzing and categorizing hydrogen bond interactions based on established criteria.

Finally, the calculated frequencies are multiplied by an anharmonicity correction factor [25–27]. The following scaling factors were chosen: 0.97 for

frequencies between 0 and 2000 cm^{-1} , and 0.95 for frequencies between 2000 and 4000 cm^{-1} .

Table 1 Classification of hydrogen bonds by bond length and energy.

Strength of hydrogen bond	Hydrogen bond length, Å	Energy, kcal/mol
Strong	2.2–2.5	14.34–28.65
Medium	2.5–3.2	3.82–14.43
Weak	3.2–4.0	Less 2.87

3 Results and Discussion

The optimized structures of betulin complexes with polar solvents (acetonitrile, isopropanol, acetone, ethanol) are presented in Fig. 4. The results of this study demonstrate that ethanol forms the shortest hydrogen bond with betulin (1.86 Å), followed by isopropanol (1.88 Å), acetone (1.90 Å), and acetonitrile (1.99 Å). These findings correlate with the experimentally observed extraction efficiencies, where ethanol yielded the highest betulin content [15]. The shorter hydrogen bond length observed for ethanol can be attributed to its ability to act as both a hydrogen bond donor and acceptor, facilitating stronger interactions with betulin. In contrast, acetonitrile, which primarily acts as a hydrogen bond acceptor, forms weaker and longer bonds due to its lower polarity and reduced ability to participate in hydrogen bonding.

The IR spectra of betulin and betulin complexes with various polar solvents such as acetonitrile, isopropanol, acetone, and ethanol are illustrated in Fig. 5. A comparison calculated IR spectrum with experimentally obtained IR spectrum data from the articles [28] reveals a high degree of correspondence. Minor variations in band shape or intensity may be attributed to differences in sample preparation (e.g., KBr pellet vs. Nujol mull), or instrumental resolution, or supramolecular effects in most cases. The main characteristic bands (OH, C–O, C–C, C=C CH_2/CH_3) match in both position and relative intensity, confirming the structural features of betulin. The calculated IR spectrum is compared with literature data or results from related triterpenoids [29] in Table 2 to validate the assignments main vibrational modes.

Since hydrogen bonds manifest as resonances in the high-frequency region of the IR spectrum, within the range from 3000 to 4000 cm^{-1} , we will investigate the spectral characteristics of betulin molecular complexes with the polar solvents in this range (Fig. 6). In the frequency range of 2800 to 3100 cm^{-1} , peaks corresponding to the vibrational frequencies of C–H bonds are observed. Peaks within the interval of 3400 to 3500 cm^{-1} are associated with the vibrations of hydrogen bonds in betulin-solvent complexes. The stretching vibrations of free O–H bonds are located in the range of 3500 to 4000 cm^{-1} .

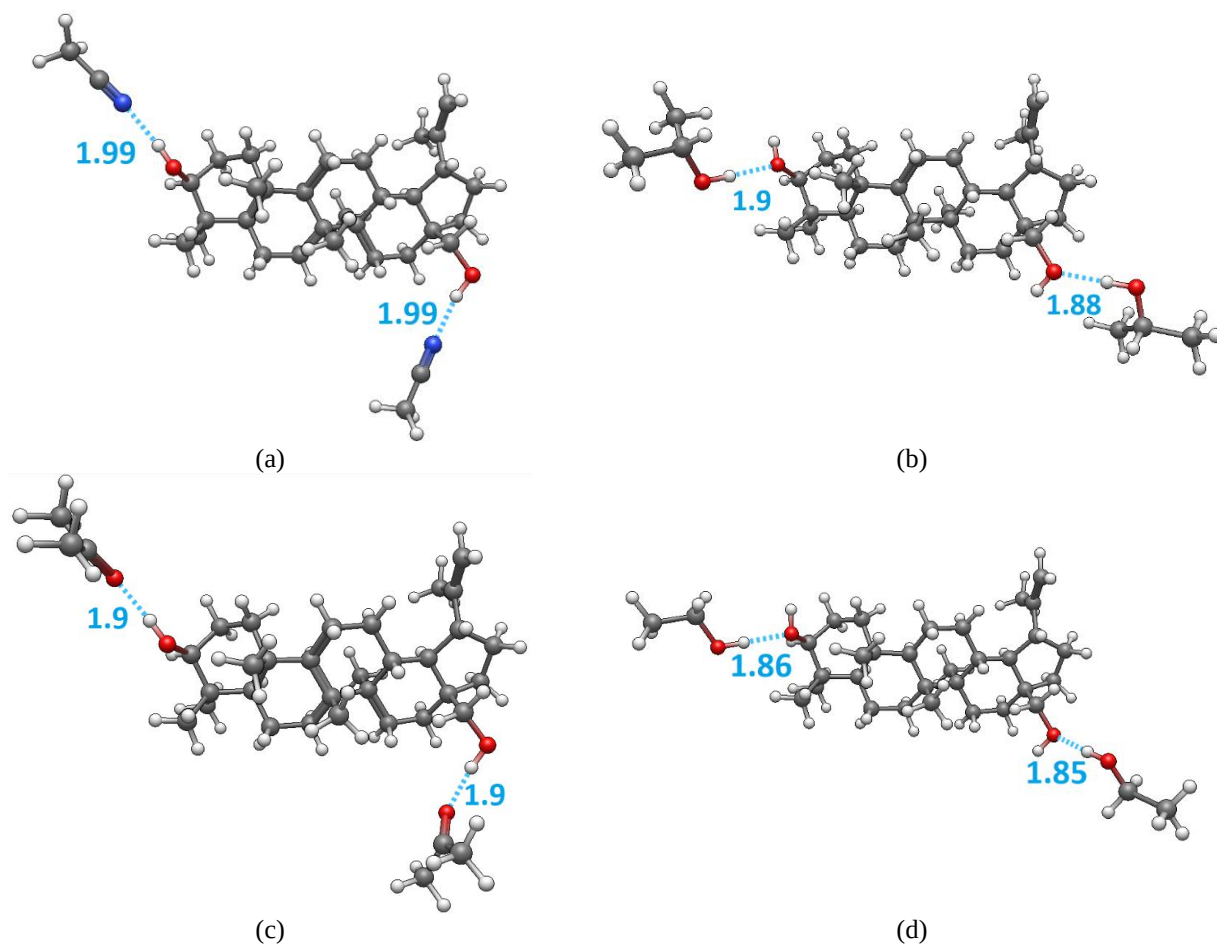


Fig. 4 Optimized structures of betulin with various solvents: (a) acetonitrile, (b) isopropanol, (c) acetone, (d) ethanol. Hydrogen bond lengths are marked in blue color.

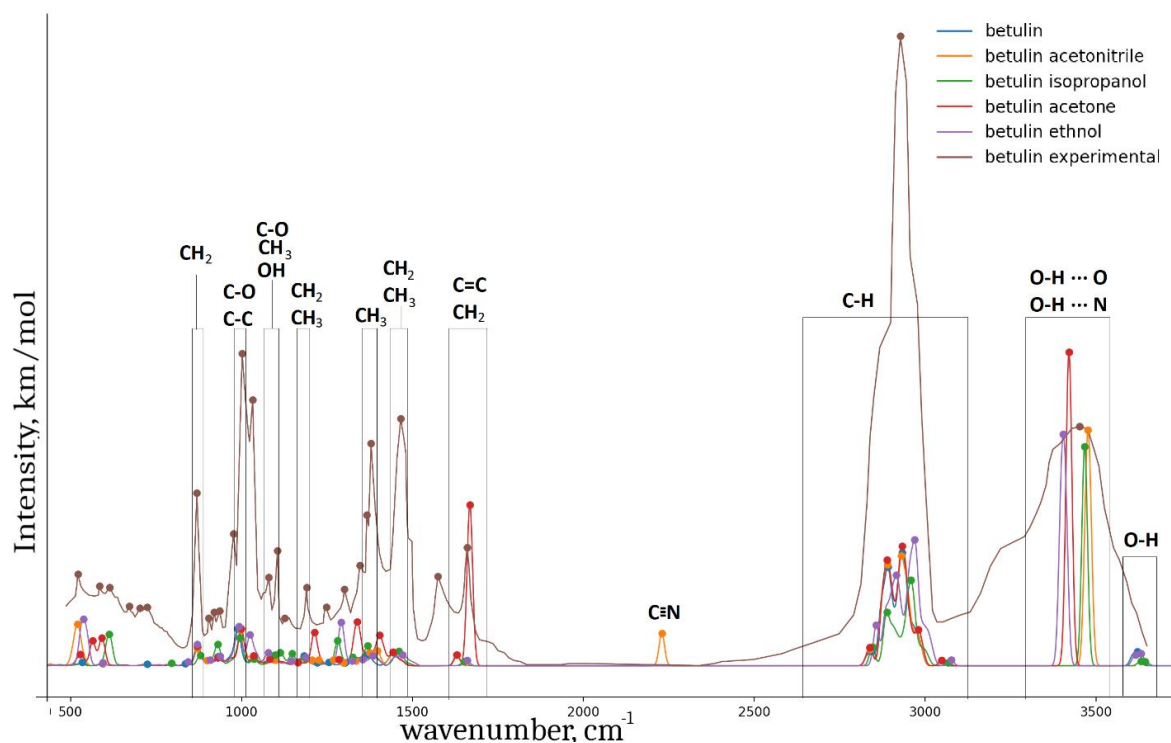


Fig. 5 IR spectra of betulin and complexes of betulin with various polar solvents (blue – betulin, orange – acetonitrile, green – isopropanol, red – acetone, purple – ethanol, brown – betulin experimental [28]).

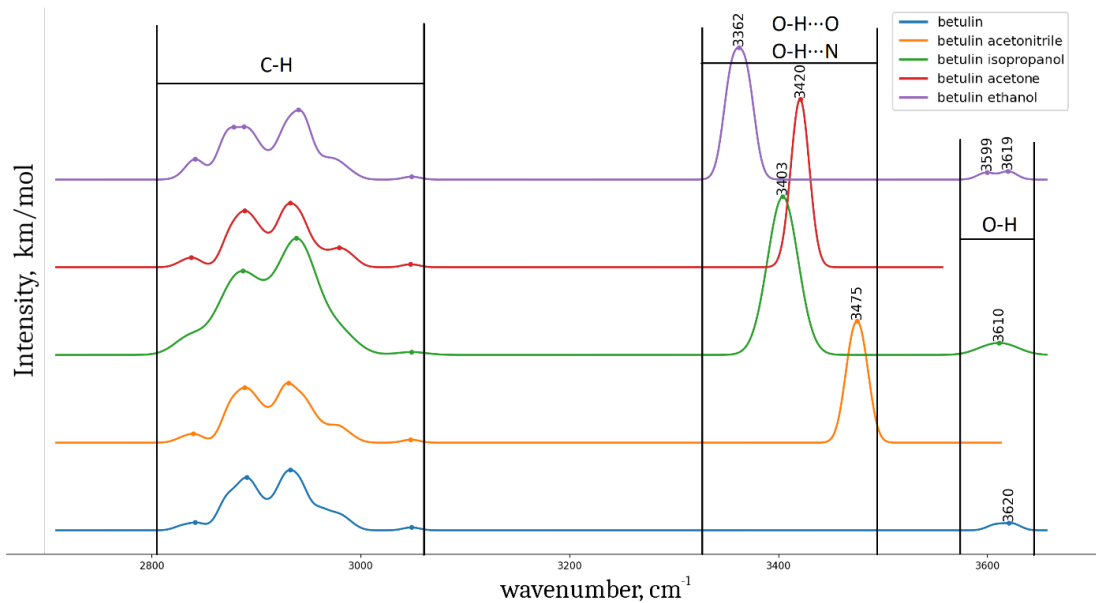


Fig. 6 High-frequency region of the IR spectra of betulin and complexes of betulin with various polar solvents (blue – betulin, orange – acetonitrile, green – isopropanol, red – acetone, purple – ethanol).

Table 2 Measured and calculated bond stretching vibrations frequencies of betulin depending on the solvent.

Bond number	Bond type	Frequency, cm ⁻¹		
		Calculated		Experiment
		Solvent	Frequency	
1	CH ₂	Acetone	870	875
		Acetonitrile, Ethanol, Isopropanol	871	
2	C–O, C–C	Acetone	1003	1006
		Acetonitrile	997	
		Ethanol	974	
		Isopropanol	981	
3	C–O, CH ₂ , OH	Acetone	1036	1032
		Acetonitrile	1036	
		Ethanol	1032	
		Isopropanol	1032	
4	CH ₂ , CH ₃	Acetone	1204	1190
		Acetonitrile	1203	
		Ethanol	1190	
		Isopropanol	1192	
5	CH ₃	Acetone, Acetonitrile	1376	1373
		Ethanol	1371	
		Isopropanol	1364	
6	CH ₃ , CH ₂	Acetone	1445, 1470	1450 1485
		Acetonitrile	1446, 1473	
		Ethanol	1445, 1474	
		Isopropanol	1449, 1474	
7	C=C, CH ₂	Acetone	1629	1642
		Acetonitrile	1630	
		Ethanol, Isopropanol	1631	

Table 3 Energy of hydrogen bonds by isolated fragment method.

Solvent name	Complex betulin, hartree	Solvents Energy, hartree	Bond energy, kcal/mol	Dipole moment, D
Acetonitrile	1587.46	132.64	17.61	3.92
Acetone	1708.13	192.97	17.45	2.84
Ethanol	1631.95	154.89	15.64	1.69
Isopropanol	1710.47	194.15	14.08	1.66

Table 4 Calculated parameters of hydrogen bonds.

Bond number	Bond type	Hydrogen bridge length, Å	Bond length, Å	Frequency, cm ⁻¹	Frequency shift Δν, cm ⁻¹	Enthalpy ΔH, kcal/mol	Intensity, km/mol
Betulin acetonitrile							
1	O–H ... N	2.96	1.99	3470	137	2.96	872
2	O–H ... N	2.97	1.99	3480	143	3.05	846
Betulin isopropanol							
1	O ... O–H	2.88	1.91	3406	228	4.12	906
2	O ... O–H	2.85	1.88	3403	245	4.3	908
Betulin acetone							
1	O–H ... O	2.87	1.9	3417	190	3.68	1042
2	O–H ... O	2.85	1.9	3424	200	3.79	1037
Betulin ethanol							
1	O ... O–H	2.84	1.86	3426	222	4.05	1124
2	O ... O–H	2.82	1.85	3440	208	3.89	1103

The results of the interaction energy calculations between betulin and its polar solvents, based on the isolated fragment method, are presented in Table 3. The highest binding energy was observed for acetonitrile (17.61 kcal/mol), while the energies for the remaining solvents were ranked as follows: acetone (17.45 kcal/mol), ethanol (15.64 kcal/mol) and isopropanol (14.08 kcal/mol).

A clear correlation with the dipole moment of the solvents was observed, indicating that the strength of the interaction is influenced by the solvent’s polarity. Specifically, acetonitrile, which has the highest dipole moment among the studied solvents, exhibited the strongest binding energy. This trend suggests that solvents with larger dipole moments facilitate stronger interactions with betulin, likely due to enhanced electrostatic forces and improved hydrogen bonding capabilities.

The intensity and frequency of the stretching vibrations of hydrogen bonds between solvents and betulin are presented in Table 4. The hydrogen bond energies were calculated using Johansen’s empirical formula.

According to the calculation results, the hydrogen bond energy of the complexes formed by various polar

solvents and betulin increases in the following order: acetonitrile (2.96 and 3.05 kcal/mol) < acetone (3.68 and 3.79 kcal/mol) < isopropanol (4.12 and 4.3 kcal/mol) < ethanol (4.05 and 3.89 kcal/mol). Using the classification criteria for hydrogen bonds presented in Table 1, the observed interactions can be characterized as moderate-strength hydrogen bonds. The trend correlates with the experimental data was taken by Levdansky et al. [15], as shown in Fig. 2. This is also evidenced by the increase in the intensity of hydrogen bond vibrations.

We propose that the empirical Johansen formula, which is based on experimental observations of characteristic frequency shifts, provides a more accurate description of the interactions between betulin and polar solvents. This is because the isolated fragment method does not account for supramolecular effects, such as collective solvent-solvent and solvent-solute interactions, which play a significant role in determining the overall binding behavior.

The findings of this study, particularly the detailed understanding of hydrogen bonding interactions between betulin and polar solvents, have significant implications for the field of biophotonics, which focuses on how light interacts with biological materials, relies heavily on the precise control of molecular interactions and the optical

properties of biomolecules. The IR spectral data, which show distinct vibrational modes for betulin-solvent complexes, could serve as a basis for developing label-free optical sensing techniques. These techniques could be used to monitor molecular interactions in real-time, providing valuable information for diagnostic applications [30].

In addition, insights into solvent interactions can inform the design of betulin-loaded nanoparticles. Nanoparticles have been extensively studied in biophotonics for their applications in imaging and therapy. By selecting solvents that form strong hydrogen bonds with betulin, such as ethanol, it is possible to enhance the encapsulation efficiency and stability of betulin within nanoparticle carriers. These nanoparticles can be engineered for targeted drug delivery, photodynamic therapy, or as contrast agents in imaging applications [31].

The results of our study on the binding energy of betulin with polar solvents provide valuable insights into the efficiency of these solvents in green extraction processes. Betulin, a naturally occurring triterpenoid, exhibits strong interactions with polar solvents due to its hydroxyl groups, which facilitate hydrogen bonding. Our findings can be extended to other structurally similar compounds, such as triterpenoids and polyphenols, which also contain polar functional groups. The reuse of polar solvents after recrystallization is a promising strategy to enhance the economic efficiency of extraction processes. Recrystallization, a common purification technique, often involves the use of polar solvents to dissolve and subsequently crystallize target compounds. After the process, the solvent can be recovered through distillation or evaporation, allowing for its reuse in subsequent extraction cycles. This not only reduces solvent waste but also minimizes the environmental footprint of the process. Studies have shown that solvent like ethanol can be effectively recycled multiple times without significant loss of efficiency, provided that proper purification steps are implemented to remove impurities [32].

Beyond extraction, polar solvents play a critical role in the synthesis and functionalization of carbon-based quantum dots (QDs), such as fullerenes and graphene quantum dots. These nanomaterials, which consist primarily of carbon, have gained significant attention for their applications in optoelectronics, bioimaging, and energy storage. The principles underlying the interactions between polar solvents and betulin, as demonstrated by our binding energy calculations, can also be extended to the synthesis and stabilization of carbon-based QDs [33]. Polar solvents, such as water and ethanol are often used as reaction media in the synthesis

of carbon-based QDs due to their ability to dissolve carbon precursors and stabilize nanoparticles during growth. For example, aqueous-phase synthesis of graphene quantum dots has been explored as a greener alternative to organic-phase synthesis, reducing the use of toxic solvents and enabling the production of biocompatible QDs for medical applications [34]. Additionally, polar solvents facilitate the functionalization of carbon-based QDs with hydrophilic ligands, enhancing their dispersibility in aqueous environments and expanding their applicability in biological systems.

4 Conclusions

As a result of the study, it can be concluded that the strongest molecular interaction, primarily driven by hydrogen bonding, occurs with ethanol. This conclusion is supported by experimental data demonstrating ethanol's superior extraction efficiency for betulin compared to other solvents. The enhanced extraction properties of ethanol can be attributed to its ability to form robust hydrogen bonds with betulin. Ethanol's polar hydroxyl group ($-OH$) facilitates the formation of these hydrogen bonds, which are critical for solubilizing and extracting betulin from the plant.

The strength of hydrogen bonding in ethanol is further evidenced by the observed increase in the intensity of vibrational modes and hydrogen bond lengths. This phenomenon underscores the significance of hydrogen bonding as a key intermolecular force in the extraction process. Additionally, ethanol's relatively small molecular size and favorable polarity enable it to penetrate the plant more effectively, thereby enhancing its interaction with betulin molecules.

Beyond its role in extraction, understanding the hydrogen bonding interactions of betulin with different solvents may also have implications for its potential use in biophotonics. The ability of betulin to form stable hydrogen-bonded complexes can influence its electronic properties, fluorescence behavior, and stability in various environments. These properties could be exploited in applications such as fluorescence-based biosensing, bioimaging, and light-mediated therapeutic strategies. Future research should explore how solvent interactions modify betulin's optical characteristics, potentially broadening its applications in biophotonics and related fields.

Disclosures

The authors declare no conflict of interest.

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Laboratory Modeling Techniques for Primary and Secondary Cataracts: Methodology and Analysis

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Abstract. This article is dedicated to the development of a laboratory methodology for modeling lens pathologies and cataract membranes of various etiologies. These models are essential for determining precise parameters of surgical or laser interventions for primary and secondary cataracts. The study explores two models based on chemical treatment leading to cataract formation. Two approaches to cataract modeling were investigated: soaking in formalin and exposure to reactive oxygen species (oxidative cataract). The optical properties of these models under laser treatment were also analyzed. It was found out that modeling intraocular lens opacification with hydrogen peroxide, a stable decline in optical response was observed during IR pulses—around 60% after 24 h, dropping to 30% after 72 h. This is attributed to increased scattering and capsule thickening. In contrast, formalin-induced capsule opacification initially caused a threefold increase in signal amplitude, likely due to increased tissue rigidity and greater displacement during heating. However, as tissue thickened, the optical response declined, returning close to baseline.

Keywords: laser; dynamics of laser radiation; primary cataract; secondary cataract; hydrogen peroxide; formalin.

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1 Introduction

Currently, research aimed at understanding the physical mechanisms and minimizing the side effects associated with laser ophthalmological surgeries is of significant relevance. The necessity for interdisciplinary approaches in this field is driven by the need to address several physical problems, such as investigating the heating processes of thin biological membranes under tension and the development of additional thermally induced laser stresses. These stresses indirectly affect the germinative (growth) zone of the lens capsule (LC), which retains proliferative activity and often initiates secondary postoperative opacification, leading to the development of so-called "secondary cataracts" (SC).

The proportion of lens pathologies within the structure of vision-disabling diseases remains high, accounting for 11–18%. Among these, the primary

clinical manifestation of lens pathology leading to visual disability following cataract surgery is complicated pseudophakia (57%), which refers to the condition after replacing the native lens with an artificial one [1, 2]. The predominant postoperative complication in cases of pseudophakia is the development of so-called "membranous" opacities in the pupillary area. These opacities vary in nature and can form both in the early postoperative period and at later stages (ranging from 10 days to over 4 years) [3–5]. The term "membranous" refers to the postoperative reduction in the transparency of the LC. Posterior LC opacification is more common than anterior opacification. The incidence of posterior LC opacification ranges from 4% to 60% of cases [5–8], while anterior LC opacification occurs in 3–16% of cases [9, 10]. Furthermore, the development of capsular contraction syndrome often results not only in LC opacification but also in deformation of the elastic

artificial lens, significantly altering the eye's optical system and refractive power. "Membranous" opacities can substantially reduce visual acuity and negatively impact quality of life, necessitating repeated interventions in some cases, either surgical or laser-based.

The frequency and severity of "membranous" opacities can be influenced by numerous factors, including age, the presence of concomitant ocular and systemic diseases, the state of the immune system and the blood-ocular barrier, the bioinertness of the artificial lens material, and its structural features [11]. The varying conditions of the LC affected by opacities dictate the strategy for laser intervention, requiring surgeons to determine the appropriate laser parameters during the procedure itself.

At present, there is no universal method for determining the optimal parameters of safe laser radiation. Consequently, each new laser intervention approach necessitates dedicated research, typically involving initial calculations, modeling of thermal field propagation and thermal stress fields, and experimental verification of the derived parameter ranges, taking into account the actual geometry and specific biological tissues. Due to the limited availability of biological tissues for experimental studies, it is often necessary to simulate both the tissues themselves and their pathological variations using various methods.

The development of cataracts can result from external adverse factors (chemical and physical), including various metabolic disorders, as well as genetic factors that affect the structure of the lens. Recently, genetic mutations disrupting the synthesis of crystallins, membrane proteins, cytoskeletal proteins, and transcription factors in the lens have been increasingly identified [12]. Among these, α -crystallin is the most important structural protein of the lens, comprising 40% of the total crystallin content in the human lens [12]. This protein regulates cell growth and genome stability, influencing both the cell membrane and the cytoskeleton. γ -crystallin is one of the most long-lived proteins, with complete turnover in humans occurring over several years [13]. Crystallins play a critical role in maintaining the lens's transparency and refractive properties. Therefore, disruption of the crystalline structure of the lens critically affects its functional properties, leading to opacification, or the formation of cataracts.

In addition to genetic disorders, oxidative stress accumulation can also contribute to lens opacification [14, 15]. Prolonged exposure to ultraviolet radiation induces photochemical production of reactive oxygen species, such as hydrogen peroxide, superoxide radicals, hydroxyl radicals, and others. These molecules gradually oxidize lens proteins, primarily crystallins, causing their aggregation and leading to loss of optical transparency. One well-documented mechanism of oxidant action is the oxidation of thiol ($-SH$) groups to sulfoxide ($-S=O$) groups, which subsequently form dimers via disulfide bridges [16]. Since hydrogen peroxide appears to be the most stable oxidant in the

progression of oxidative cataracts [17, 18], modeling oxidative cataracts has been conducted using hydrogen peroxide solutions of varying concentrations.

Since the literature on oxidative cataracts primarily focuses on cataract modeling in the lenses of small animals (such as rats [19] and rabbits [20]), this study required selecting appropriate concentrations of solutions and exposure times for lens capsules of larger animals. This was done to modify the biological tissue to induce cataracts without causing capsule destruction. Limited studies on the application of oxidative techniques to pig eyes have demonstrated the success of this approach [21]. However, in our study, it was crucial to optimize tissue treatment to develop two distinct methods for laboratory cataract modeling with following laser exposure.

Thus, the task of simulating processes that lead to postoperative opacities of LC of various types, as well as modeling the properties of SC with subsequent laser treatment of modified biological tissue samples, is of significant relevance.

This article is dedicated to the development of a laboratory methodology for modeling lens pathologies and cataract membranes of various etiologies for primary and SC. The study explores two models based on chemical treatment leading to cataract formation. Two approaches to cataract modeling were investigated: soaking in formalin and exposure to reactive oxygen species (oxidative cataract). The optical properties of these models under laser treatment were also analyzed.

2 Materials and Methods

2.1 Laboratory Methodology for Oxidative Cataract Modeling

The pig lenses obtained from a slaughterhouse were used in the experiment. The extracted eyes were stored in a refrigerator at 4 °C in 0.9% NaCl physiological solution for no more than 24 h.

The oxidative solutions were based on a phosphate-buffered saline (PBS) with a pH of 7.4, prepared using the following composition: 6 g of $Na_2HPO_4 \cdot 12H_2O$, 0.19 g of K_2HPO_4 , and 8 g of NaCl dissolved in distilled water. The pH was measured using Universal test paper, and the solution volume was adjusted to 1000 ml. The final pH corresponded to 7 on the indicator scale. Hydrogen peroxide (H_2O_2) solution was used to initiate oxidative processes in the lens tissue, with the peroxide concentration varying according to Table 1.

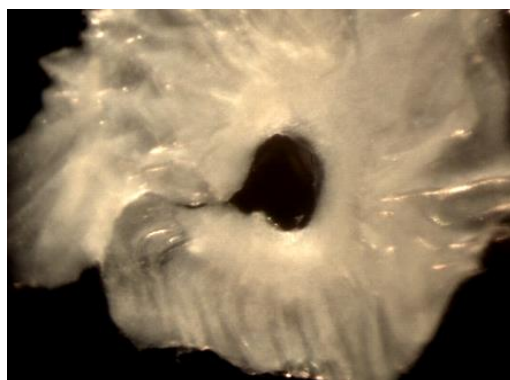
Immediately before the experiment, lenses were extracted from the eye samples, and their transparency was assessed. The lenses were then placed in PBS containing hydrogen peroxide. The reaction was conducted in a room without exposure to daylight. The changes were recorded on photo and video. The lenses were incubated for 72 h with monitoring at 24, 48, and 72-h intervals. Visual observations were recorded using a Celestron Amoeba Microscope (Model 44325). The experiment was carried out on a minimum of six samples at each point.

Table 1 Volumes of PBS and 3% hydrogen peroxide required to prepare solutions of the indicated concentrations.

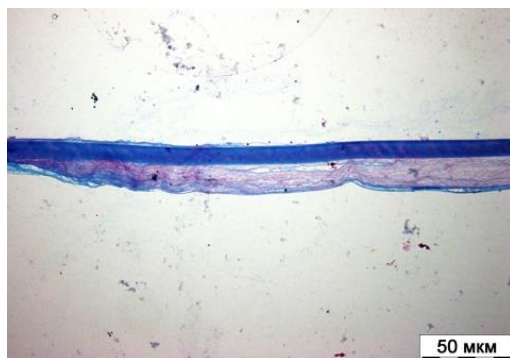
Concentration	PBS	H ₂ O ₂ 3%
4 mM	20 ml	0,1 ml
8 mM	20 ml	0.2 ml
13 mM	20 ml	0.3 ml

2.2 Laboratory Methodology for Modeling Cataractous Formations Using Formalin

The experimental model for LC opacification using formalin was based on the results from previous comprehensive studies of autopsy LC samples in pseudophakic eyes (with artificial lenses) exhibiting SC with varying optical densities [22]. The density of these dissected samples subsequently influenced the parameters for laser tissue ablation [23]. LC opacities were categorized as follows: SC with dense fibrous deposits on the internal surface of the posterior lens capsule (PLC) were classified as “hard” membranes (Fig. 1).



(a)



(b)

Fig. 1 (a) Macroscopic view of a sample of a “hard” posterior capsule (PC): an optically dense capsule with a shiny outer surface and a wavy relief, magnification $\times 1.2$. (b) Morphological structure of a “hard” PC sample [24]: fibrous tissue on the inner surface of the

PC. Wrinkling of the PC is observed. Semi-thin section stained with methylene blue and basic Fuchsin.

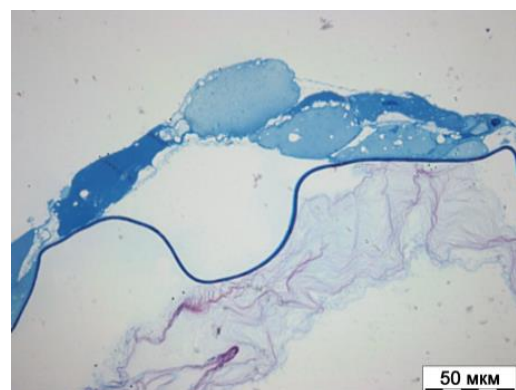
SC films with deposits of pseudoregenerative cellular components (Elschnig-Adamiuk pearls) on the inner surface of the posterior capsule were classified as “soft” films (Fig. 2).

This formalin-based method for modeling LC pathological changes was chosen to densify the tissue and create cross-links, as this approach is commonly employed by ophthalmologists to simulate cataracts and practice surgical skills for removing cataractous opacities [25]. It is also used for studying tissue properties during soaking in the solution [26].

The experiment was conducted over 60 min, with control intervals at 15, 30, and 60 min, with minimum three samples in every point. It was hypothesized that varying exposure times would result in LC opacities of differing densities (softer or harder), enabling the creation of conditions as close as possible to the actual scenarios described earlier. Visual observations were recorded using a Celestron Amoeba Microscope (Model 44325).



(a)



(b)

Fig. 2 (a) Macroscopic view of a “soft” PC sample: uneven optical density of the capsule (areas of transparency alternate with denser regions), magnification $\times 1.2$. (b) Morphological structure of a “soft” PC sample: Elschnig-Adamiuk pearls on the inner surface of the PC. Semi-thin section stained with methylene blue and basic Fuchsin.

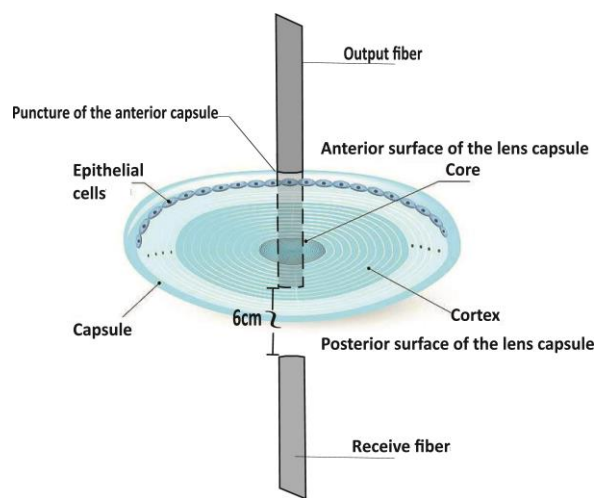


Fig. 3 Optical method for studying the dynamics of transmission of probing laser radiation [23], the diagram of the experiment is shown in cross section.

2.3 Optical Method for Investigating the Dynamics of Transmission of Probing Laser Radiation

Experiments on the study of the transmission dynamics of low-intensity probing laser radiation were carried out. The laser radiation penetrated a sample of a whole lens perpendicular to its surface using an optical fiber. Behind

the surface of the sample, coaxially with the input optical fiber supplying the radiation, there was a “receiving” optical fiber connected to an optical multichannel analyzer in which the signal was digitized (Fig. 3). Peroxide soaking was conducted for 72 h with control intervals at 24, 48, and 72 h. Formalin soaking was conducted for 60 min with control intervals at 15, 30, and 60 min. The geometry and power of the visible radiation were consistent across all four irradiation series of each modeling process. The wavelength of the constant probing radiation was 0.53 μm (visible spectrum). Heating irradiation with a wavelength of 1.56 μm was performed simultaneously with a probing one in a pulsed-periodic mode, with a pulse duration of 100 ms and a pause of 1900 ms between pulses. The total irradiation time was 20 s (10 pulses), with power levels of 0.3 W and 0.5 W.

3 Results and Discussion

3.1 Visual Assessment of Cataract Modeling

Microscopic analysis conducted with the Amoeba Celestron Microscope 44325 revealed distinct patterns in cataract formation for oxidative cataracts (Table 2). The lens soaked in PBS solution showed negligible changes in light transmission within the visible spectrum after 24 h. However, at 48 and 72 h, structural opacities and damage began to appear.

Table 2 Visual assessment of lenses subjected to various modifications using hydrogen peroxide.

Time, h	0	24	48	72
PBS				
PBS + H2O2 3% 4 mM				
PBS + H2O2 3% 8 mM				
PBS + H2O2 3% 13 mM				

Table 3 Visual assessment of lenses subjected to various modifications using formalin [23].

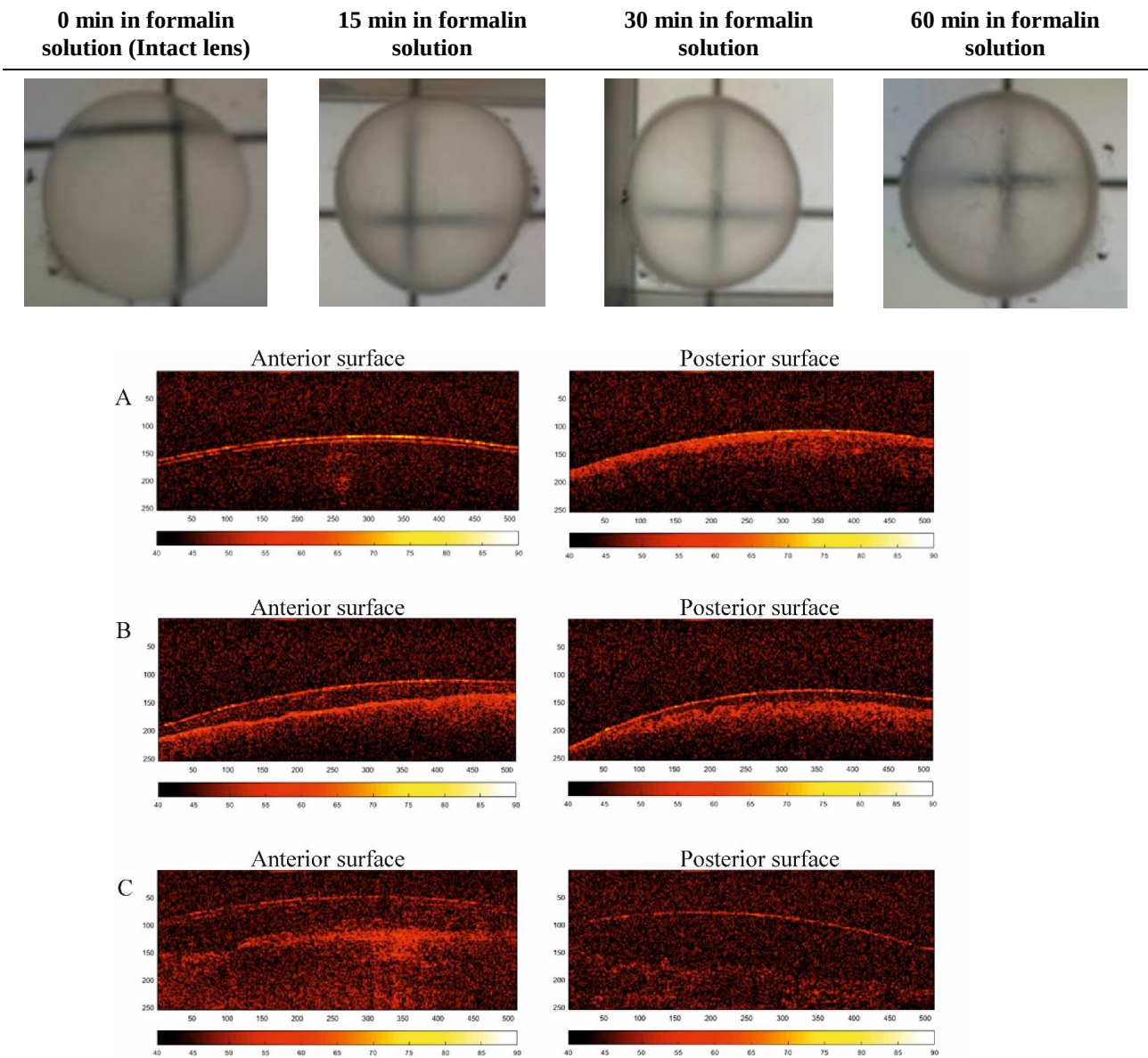


Fig. 4 OCT structural image of (A) the intact lens of the eye, no more than 4 h have passed since the material was extracted, (B) the eye lens 30 min after placing them in formalin, (C) the lens during modeling of oxidative cataract in PBS + 8 mM peroxide solution for 48 h.

The addition of hydrogen peroxide at varying concentrations had differential effects on structural changes and lens opacity. A 4 mM concentration led to noticeable separation of the capsule and body only after 72 h, whereas a 13 mM concentration caused clear structural separation as early as 24 h. However, at 13 mM and 24 h, frequent detachment of the lens capsule from its body was observed during sample handling, and prolonging exposure to 72 h was deemed unnecessary due to time constraints. Therefore, a concentration of 8 mM with an exposure time of 48 h was selected, achieving distinct structural separation in the lens without capsule detachment.

In formalin-based modeling, lens opacity became evident within 15 min of soaking (Table 3). Furthermore,

the opacity appeared to affect only the superficial layers, as the peripheral band of the lens visibly thickened. This observation suggests the potential to model SC films, which are a common reason for laser capsulotomy procedures in cases of SC.

3.2 OCT Visualization of Lens Structure

The evaluation was performed on both sides of the lens (anterior and posterior surfaces) along the axial line (Fig. 4). For intact lens tissues, OCT imaging revealed a structural separation in the superficial layers of the anterior capsule, with a very thin gap between them, while no such separation was observed on the posterior surface.

Soaking the lens in formalin for 30 min or longer induced the following structural changes observed on OCT images:

- Anterior surface: in 60% of cases, there was a significant increase in the distance between the capsule (surface) and the lens body (cortical layers and nucleus). In the remaining cases, an increase in this distance was also observed, but it was less clear.
- Posterior surface: distinct separation of the surface was observed in only 30% of cases.

It is important to note that this pattern emerged as early as 15 min of formalin exposure and remained consistent regardless of the duration of soaking.

In the case of oxidative cataract modeling, OCT images revealed changes occurring within the lens body rather than on its surface, as seen with formalin treatment.

These OCT imaging results confirm the conclusions drawn from the visual assessment of lens changes during cataract modeling. Soaking in formalin primarily affects the superficial layers of the lens (its capsule), while oxidative cataract modeling leads to structural alterations within the lens body.

3.3 Results of the Study on the Dynamics of Transmission of Probing Laser Radiation

The main point of this study was to obtain information about optical response of lens to IR heating pulses. Data presented in Fig. 5 shows the average relative change of the constant visual signal due to periodical IR heating pulses for lens with varying exposure durations in various solutions. At least 3 samples were used to plot each bar in Fig. 5.

Using hydrogen peroxide modeling the opacification of the intraocular lens, a stable decrease in the relative amplitude of the change in the transmitted probing radiation during heating IR laser pulses is noted depending on the time of exposure to the solution. The intensity of optical response was around 60% for 24 h of solution influence in comparison to initial signal and decreases further down to 30% for 72 h. This is due to the general decrease in optical transparency due to the increase in scattering of the capsule and its thickening.

At the same time, modeling the opacification of the capsule using formalin, an initial significant increase up to 3 times in the amplitude of the change in the visible signal is noted. This behavior can be explained by a change in the mechanical properties of the capsule surface. Formalin increases the rigidity of the tissue, which leads to a greater spatial shift of the sample as a reaction to thermal laser exposure. Subsequently, an increase in the thickness of the tissue again leads to a decrease in the optical response and almost returns to its initial value.

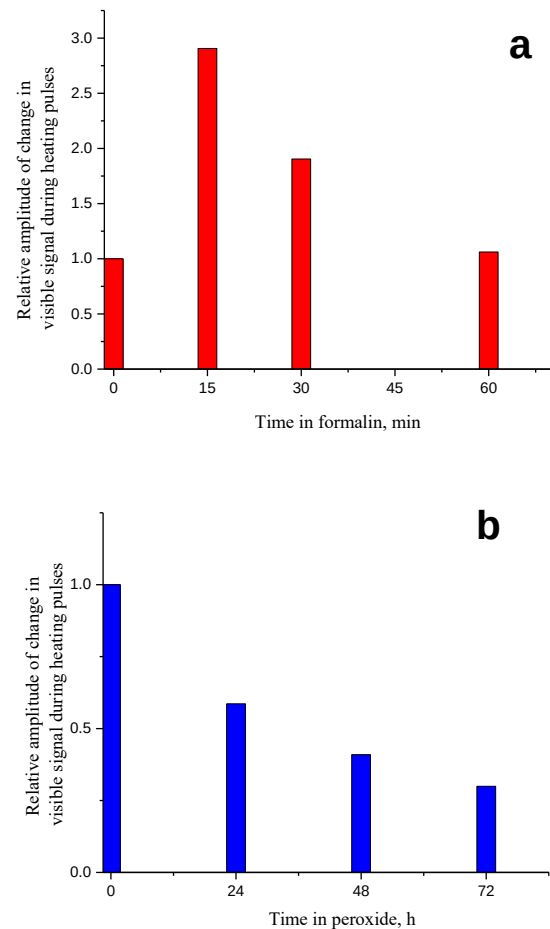


Fig. 5 Average relative amplitude of change in visible probing signal due to IR heating pulses for the samples with varying exposure durations in (a) formalin and (b) hydrogen peroxide.

4 Conclusions

A comparative study of two methods for creating models of pathologies in the “lens + capsule” system was conducted, corresponding to two of the most common forms of intraocular lens opacification that reduce vision and require surgical or laser treatment. These methods were tested on isolated eyes of large laboratory animals.

In the oxidative cataract model, changes occur within the lens body itself: the optical response of the lens mass alters, and its density changes. This effect on the density of the lens mass can be used to create cataract models for developing laser parameters aimed at fragmenting and removing lens material during lens replacement surgery. When modeling intraocular lens opacification with hydrogen peroxide, a stable decline in optical response was observed during IR irradiation – around 60% after 24 h, dropping to 30% after 72 h. This is attributed to increased scattering and capsule thickening, which demonstrates the model’s suitability for simulating the initial stages of cataract formation.

In contrast, formalin-induced capsule opacification initially caused a threefold increase in signal amplitude

change, likely due to increased tissue rigidity and greater displacement during heating. However, as tissue thickened, the optical response declined, returning close to baseline. In the formalin-induced cataract model, the changes are localized to the superficial layers of the lens (its capsule).

This approach allows for the modeling of two types of cataract films. These models can subsequently be used to refine photodestruction laser mode for treating SC films in eyes with artificial lenses.

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Disclosures

The authors declare that they have no conflict of interest.

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Analysis of Pulse Wave Morphology Measured by Photoplethysmography During Arm Position Changes

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Abstract. Numerous studies underscore the importance of analyzing pulse wave morphology via photoplethysmography (PPG) as an accessible method for investigating localized vascular responses. Contemporary approaches to modeling pulse wave morphology are based on the assumption that the dicrotic wave arises from reflections at vascular bifurcations and other vascular inhomogeneities. An alternative perspective posits that normal blood circulation is sustained through smooth muscle tone. To validate this hypothesis, we analyzed pulse wave morphology during arm position changes, which are associated with alterations in arterial tone within the vascular system. This study substantiates the feasibility of evaluating vascular resistance changes through dicrotic wave curvature during arm repositioning, measurable via the slope angle of the PPG signal at this segment. Furthermore, it is shown that the venulo-arteriolar reflex triggered by arm position changes leads to ambiguous dynamics in pulse wave amplitude variations. The presence or absence of the venulo-arteriolar reflex impacts pulse wave amplitude but does not affect dicrotic wave curvature, which is exclusively governed by changes in arteriolar smooth muscle tone.

Keywords: pulse wave morphology; photoplethysmography; diastolic wave; dicrotic wave; venulo-arteriolar reflex; vascular resistance.

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1 Introduction

The pulse wave morphology carries information about vascular atherosclerosis, arterial hypertension, diabetes mellitus [1–3], and endothelial dysfunction, which serves as a predictor of cardiovascular disease risk [4]. Greatest interest lies in studying pulse wave morphology using non-invasive methods such as rheography, photoplethysmography (PPG), and sphygmography. Key parameters include: the ratio of systolic to diastolic peak amplitudes, the area under the pulse wave curve, the ratio of areas before and after the dicrotic wave, the ratio of pulse wave width to systolic amplitude, the amplitude of the reflected wave (augmentation index), and others [5]. Currently, photoplethysmography (PPG), as a simple and accessible method, is widely used for developing diagnostic approaches to assess the cardiovascular system. The PPG signal is divided into pulsatile and non-

pulsatile components [6, 7]. The pulsatile component of the PPG signal consists of a systolic phase (anacrotic phase), corresponding to increased blood volume in the microcirculatory bed due to cardiac ejection, and a diastolic phase (catacrotic phase), representing blood redistribution in the microcirculatory bed. The systolic peak is identified at the apex of the anacrotic phase, where systolic blood ejection is maximal. The minimum trough on the diastolic segment is defined as the dicrotic notch (incisura), and the subsequent peak is termed the dicrotic rise [8].

Numerous studies highlight PPG's utility as a cost-effective tool for analyzing localized vascular responses. However, localized pulse wave analysis for assessing specific arterial segments is not yet recommended, as such measurements lack validation in prospective studies for predicting cardiovascular disease (CVD) outcomes. This limitation has excluded

PPG and other pulse wave analysis methods from the Russian Ministry of Health's clinical guideline classification system.

Modern model concepts of pressure pulse wave formation [9] and proportional changes in PPG signal amplitude [10] are based on the theory of reflected wave existence [5]. However, there is currently no consensus on the mechanism of reflected wave formation. Specifically, modern textbooks and monographs state that "the secondary dicrotic wave (rise) is caused by blood reflection from closed valves" [11–13]. This claim is no longer repeated in ultrasound diagnostics literature, as the dicrotic segment in Doppler ultrasound is identified as retrograde blood flow [14, 15].

A significant body of research assumes that the reflected wave originates from vascular bifurcations and other vascular inhomogeneities [16–20]. In Ref. [21], it is proposed to consider reflected wave formation as a combination of reflections from the ends of branching arterial networks. Identifying the exact site of reflected wave generation remains controversial. For example, it is argued that the most prominent reflection occurs at the bifurcation of the descending abdominal aorta [5]. If this is accepted, the reflected wave should propagate in the direction of systolic blood ejection into peripheral arm vessels, whereas Doppler ultrasound reveals retrograde flow.

With aging, the diastolic pulse wave morphology becomes smoother, and the dicrotic wave shifts toward the systolic peak. Blood flow dynamics in elderly subjects are influenced by at least two factors: increased stiffness of arterial vessel walls and reduced smooth muscle tone in arterioles. These factors explain age-related changes in pulse wave morphology: increased stiffness leads to earlier retrograde flow waves, while reduced smooth muscle tone diminishes dicrotic wave prominence.

A second debated issue involves the venulo-arteriolar reflex to explain the observed increase in PPG signal amplitude during arm elevation above heart level. Raising the arm reduces arterial and venous blood volume in the limb, as recorded by laser Doppler flowmetry, which measures both pulsatile and steady components [22].

Several studies note that reduced blood volume decreases PPG signal amplitude [20, 23–25]. For instance, Ref. [26] demonstrated through anesthesia, sympathetic activation, and vasoconstrictor studies that peripheral vasodilation increases systolic peak amplitude, while vasoconstriction reduces it. Thus, arm elevation and reduced blood volume should decrease pulse wave amplitude. However, the inverse trend observed in Refs. [20, 27, 28] is attributed to the venulo-arteriolar reflex. As shown below, this pattern is not universal. Despite complex explanations, authors consistently emphasize the need for further research on this reflex.

Despite ambiguity in the reflected wave mechanism, pulse wave morphology remains widely used for vascular disease diagnostics. An alternative perspective posits that

normal blood circulation is maintained by smooth muscle tone. To validate this, we propose analyzing pulse wave morphology during arm repositioning, which directly alters arterial vascular tone. We also discuss the venulo-arteriolar reflex during positional changes, demonstrating that PPG amplitude variations are not universally consistent.

2 Materials and Methods

The study was conducted on a group of volunteers comprising 10 relatively healthy young males aged 21–28 years. Exclusion criteria included tobacco smoking, arterial hypertension, other cardiovascular diseases (CVDs), diabetes mellitus, acute respiratory viral infections (ARVIs), and the use of vasoactive medications.

The experiment was performed in a room maintained at 23 ± 1 °C, with participants in a seated position. Prior to blood flow signal recording, participants remained seated for at least 5 min with their arm resting on a horizontal support at heart level. For each subject, three sequential recordings were performed with the right arm (equipped with the PPG sensor) in different positions:

1. Baseline recording (middle arm position): the arm rested relaxed and flexed on a horizontal support at heart level.
2. Lower arm position recording: the arm hung freely downward.
3. Upper arm position recording: the hand rested on a horizontal support to minimize muscular strain.

Arm repositioning took approximately 30–40 s, allowing PPG signal amplitude and morphology to stabilize. After a 10-s recording in the second (lower) position, the arm was moved to the third (upper) position by vertical elevation at the elbow joint. The total duration in the second position did not exceed one minute. The third PPG recording began after an additional 30–40 s.

This protocol was designed to study the vascular system's rapid response to gravity-induced blood flow changes. Additionally, prolonged arm positioning was avoided to optimize practicality for developing a screening procedure assessing vascular reactions to positional changes. A PPG device, Easy Pulse (v.1.0), was placed on the distal phalanx of the right middle finger. The device operates by detecting changes in blood volume within finger tissues using a 950 nm light source and a photodetector from the HRM-2511E sensor (Kyoto Electronic Co., China). Signal acquisition involved multiple stages. The photodetector signal was first passed through a passive high-pass RC filter to block the DC component of the PPG signal within the 0.5–10 Hz frequency range. Simultaneous low-pass filtering and amplification of the analog signal were performed using the MCP6004 operational amplifier. The amplified signal was then digitized by an NI ELVIS device (National Instruments, USA) and stored in the computer's persistent memory for subsequent processing.

3 PPG Signal Measurement and Processing Technique

Fig. 1 shows the PPG pulse wave of a 22-year-old volunteer recorded over 10 s with the arm positioned downward (–).

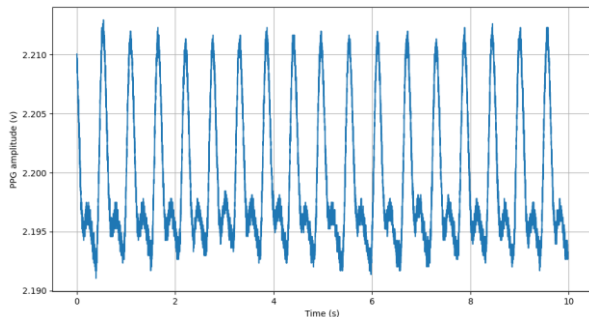


Fig. 1 PPG pulse wave of a 22-year-old volunteer with the arm positioned downward (–), recorded over 10 s.

Subsequent processing involved isolating cardiac cycles and normalizing pulse waves to unit amplitude (Fig. 2a). Unlike earlier methodologies, we selected the curvature of the diastolic segment on the dicrotic wave (specifically, the transition from the dicrotic notch [incisura] to the dicrotic peak) as the informative parameter.

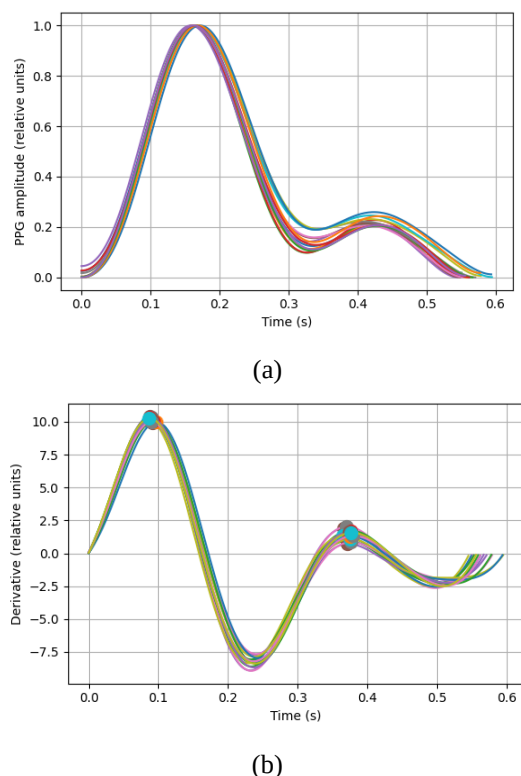


Fig. 2 Isolation of cardiac cycles, normalization of pulse waves to unit amplitude, and first derivative of the pulse wave: (a) normalized cardiac cycles of the PPG pulse wave; (b) corresponding derivative waveforms with maxima on the pulse wave.

To quantify dicrotic wave curvature, the first derivative was computed for each cardiac cycle (Fig. 2b). Colored circles mark derivative maxima corresponding to the transition from the incisura to the dicrotic peak.

Signal processing and analysis were performed using the Python programming language and Jupyter Notebook. Raw pulse wave signals were filtered with a 3rd-order Butterworth low-pass filter (passband: 0–6 Hz). Amplitude normalization scaled the signal to the [0, 1] range. Peak detection was performed using the *scipy.signal* library and the *argrelextrema* function, while derivatives were calculated using the *diff* method of the *numpy* library. The tangent of the slope angle ($\tan\alpha$) at the extremum point was computed, where α represents the maximum PPG slope angle during the incisura-to-dicrotic-peak transition.

Fig. 3 displays the variability of maximum PPG slope angles derived from dicrotic wave derivative maxima (Fig. 2b). This variability reflects spontaneous vascular tone regulation mechanisms.

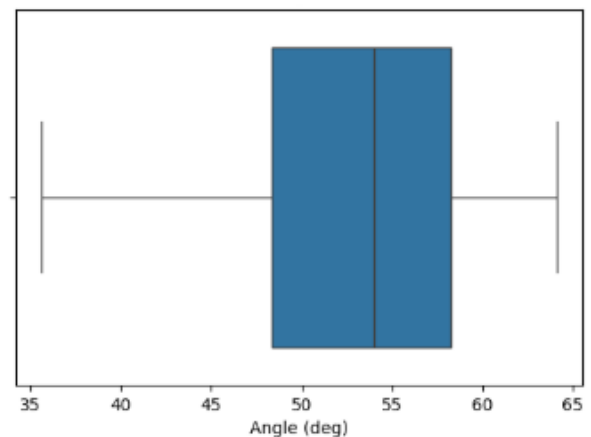


Fig. 3 Spread of derivative values in the dicrotic wave region for the pulse wave shown in Fig. 2.

PPG measurements were taken in seated participants under three arm positions: (–) below heart level (arm hanging vertically downward), (0) at heart level (arm resting freely on a table), (+) above heart level (arm elevated, supported at the elbow). These positions minimized myogenic influences from skeletal muscles on vascular walls [29].

Fig. 4 illustrates diastolic PPG segments (10-s recordings) and their corresponding derivative waveforms with dicrotic wave maxima. Averaged PPG amplitudes were: (a, b) 0.085 V (position –), (c, d) 0.038 V (position 0), (e, f) 0.018 V (position +). Maximum PPG slope angles, calculated from derivative peaks, averaged: (a, b) -16° (position –), (c, d) $+27^\circ$ (position 0), (e, f) $+53^\circ$ (position +).

As shown in Fig. 4, the mean dicrotic wave slope angle increases as the arm shifts from above (+) to below (–) heart level, ranging from -16° to $+53^\circ$. Notably, the dicrotic wave region remained stationary relative to the systolic peak (anacrotic apex).

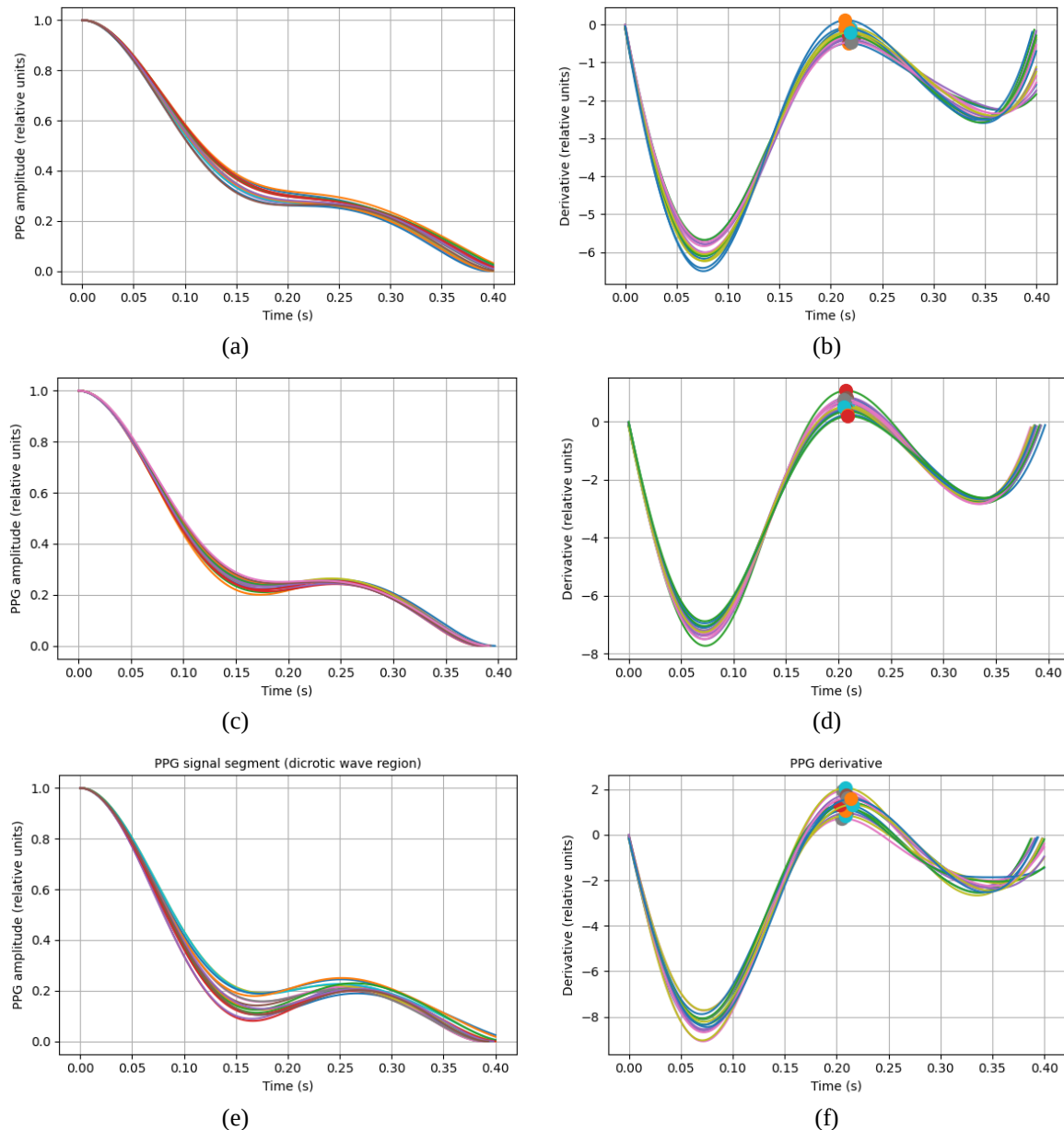


Fig. 4 (a, c, e) Normalized diastolic PPG segments (10-s recording) from a volunteer in three arm positions and (b, d, f) their corresponding derivative waveforms with maxima in the dicrotic wave region: (a, b) (+), (c, d) (0), (e, f) (-).

4 Results of the Study

Fig. 5 shows the calculated maximum PPG slope angles on the dicrotic wave segment for 10 volunteers across three arm positions: (-) below heart level, (0) at heart level, and (+) above heart level. Fig. 6 displays the spread of PPG slope angles for the same group. As seen in Figs. 5 and 6, lowering the arm from position (+) to (-) resulted in an increase in the dicrotic wave slope angle, corresponding to elevated arterial resistance.

Figs. 7 and 8 present the PPG signal amplitudes and their variability for 10 volunteers under the three arm positions: (+) above heart level, (0) at heart level, and (-) below heart level. Fig. 7 reveals that the relationship between PPG amplitude and arm position varies among individuals: amplitudes may increase, decrease, or peak at heart level. Fig. 8 demonstrates that, on average, the

PPG amplitude peaks when the arm is positioned at heart level.

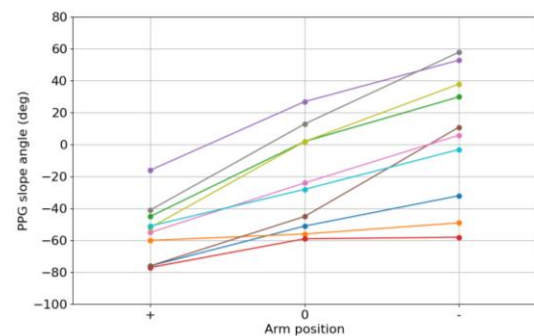


Fig. 5 PPG slope angle values on the dicrotic wave segment for 10 volunteers across three arm positions.

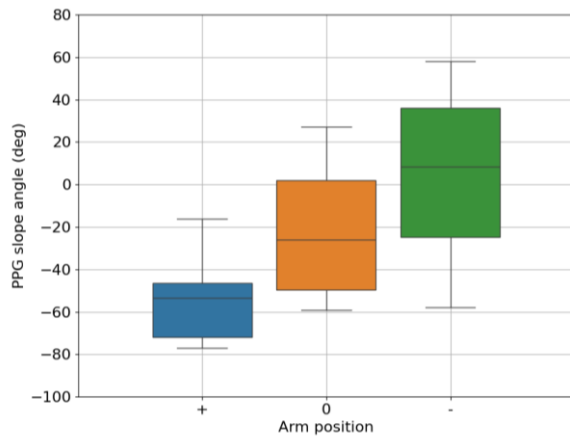


Fig. 6 Box-plot of spread of PPG slope angles on the dicrotic wave segment for 10 volunteers across three arm positions.

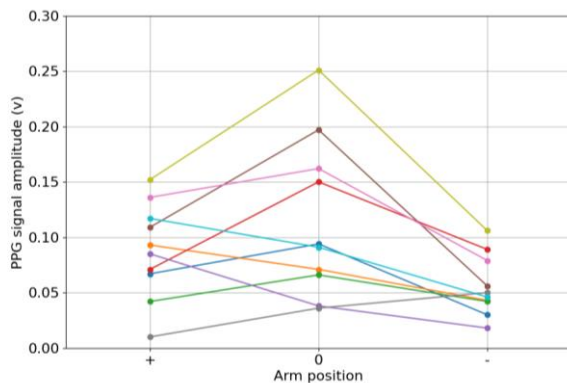


Fig. 7 PPG signal amplitude values for 10 volunteers across three arm positions.

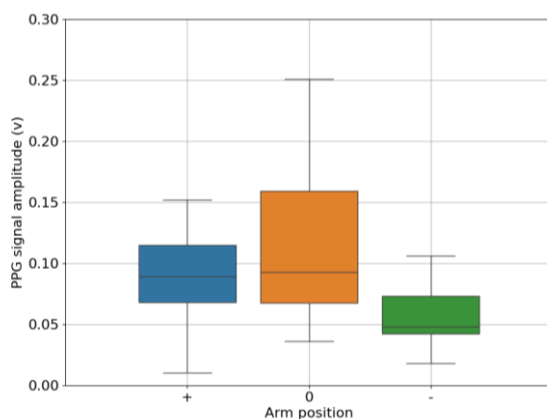


Fig. 8 Box-plot of PPG signal variability values for 10 volunteers across three arm positions.

This variability in PPG amplitude dependence on arm position aligns with existing literature and is likely associated with the type and state of the autonomic nervous system in individual volunteers. However, the autonomic nervous system's influence on PPG waveform morphology appears less pronounced, as the pattern of its change is more consistent and clearly defined.

5 Discussion

During arm repositioning, we measured changes in two parameters: systolic wave amplitude and pulse wave curvature at the dicrotic segment, calculated from the PPG slope angle at this segment. It has been shown that the dependence of systolic wave amplitude on arm position relative to heart level can exhibit various patterns: increasing, decreasing, or peaking at heart level (Fig. 8).

Most studies investigating pulse wave amplitude during arm repositioning describe a uniform trend: increased amplitude during arm elevation and decreased amplitude during lowering [20, 27, 28]. These studies utilized an extended, supported arm position and measurements were carried out 2–5 min or more minutes after repositioning. The age of the participants was usually over 30 years old. Observed perfusion changes were attributed to venous pressure variations and the venulo-arteriolar reflex.

The article by Z. Ovadia-Blechman et al. [29] reports a non-monotonic systolic amplitude dependence on arm position, consistent with the trends shown in Fig. 8.

Our prior work revealed that the venulo-arteriolar reflex's manifestation depends on age and duration of arm positioning [30]. This reflex is most pronounced in elderly individuals, whereas autonomic activity dominates in younger cohorts. These factors explain the variable systolic amplitude dependence on arm position: increasing, decreasing, or peaking at heart level (Fig. 8).

In our protocol, recordings began 30–40 s after repositioning, followed by a 10-s PPG acquisition. This design captured rapid vascular responses to gravity-induced hemodynamic shifts. Resting the arm on a table in a flexed position minimized muscular tension and venulo-arteriolar reflex interference. However, suppressing sympathetic activity in young participants proved impossible, as confirmed by behavioral observations. We did not account for height, weight, autonomic dysfunction, or baseline finger temperature – factors potentially influencing reflex dynamics and amplitude trends.

The second observed pattern involved altered arterial resistance shaping the dicrotic wave during arm repositioning. This study pioneers explaining pulse wave morphology dynamics via arteriolar smooth muscle tone changes in the microcirculatory bed, despite prior reports of dicrotic wave attenuation during positional shifts.

For instance, Ref. [20] (Fig. 6) illustrates pulse wave morphology changes during arm repositioning, concluding that elevated arm positions increase vascular resistance. This conclusion, drawn from empirical data across age groups, athletes, and functional tests [9, 20, 31–33], assumes unchanged arm positioning during measurements.

Abandoning the “reflected wave” concept and focusing on smooth muscle tone yields opposing conclusions. Elevated arms exhibit prolonged systolic rise and decay times [20]. Reduced blood volume delays arterial filling, while decreased pressure lowers resistive vessel tone. Retrograde flow during diastole is also

delayed, widening the diastolic wave. As the dicrotic peak's timing remains stable ([20] and our results), its vertical rise increases the reflection index (RI) despite diminished retrograde flow.

Studies [20, 33] claim that “arterial and arteriolar resistance should not significantly affect PPG morphology during arm elevation, with venous resistance dominating over arterial”. This contradicts Doppler ultrasound findings.

Dicrotic wave formation on the diastolic segment arises from retrograde flow, detectable via ultrasound Doppler [14, 15, 34]. Vascular resistance calculations rely on retrograde-to-antegrade flow ratios, with retrograde flow magnitude linked to smooth muscle tone [35, 36]. Thus, steeper systolic decay and dicrotic rise slopes correspond to increased resistance and arteriolar tone.

Arm repositioning alters vascular tone [22, 30, 37], modifying pulse wave curvature. The venulo-arteriolar reflex impacts amplitude but not dicrotic curvature, which is exclusively governed by arteriolar (not venular or anastomotic) smooth muscle tone [38, 39].

6 Conclusions

The functional test involving arm elevation or depression alters systolic wave amplitude and dicrotic wave curvature. We demonstrate that the dependence of systolic amplitude on arm position relative to heart level can vary: increasing, decreasing, or peaking at heart level. This hemodynamic variability may stem from age- and time-dependent manifestations of the venulo-arteriolar reflex.

In our study, blood flow signals in young volunteers were recorded 30–40 s after arm repositioning, followed by a 10-s PPG acquisition. Resting the flexed arm on a table at heart level minimized muscular tension and venulo-arteriolar reflex interference, though the reflex's presence/absence remained unconfirmed.

Numerous studies on diastolic pulse wave morphology highlight the lack of standardized PPG-

based vascular assessment methods, leaving the mechanisms of incisura and dicrotic rise formation unclear.

Key evidence for reduced arterial tone during arm elevation lies in altered dicrotic wave morphology. We observed decreased dicrotic curvature and slope angle during arm elevation, with no significant shift in diastolic peak timing relative to the systolic peak.

Current pulse wave models assume systolic components arise from forward pressure waves propagating from the left ventricle to the periphery, while diastolic components originate from pressure waves reflected back from peripheral arterioles. The term “reflected wave” misrepresents retrograde flow dynamics and complicates interpretation. Our approach attributes dicrotic wave formation to arteriolar smooth muscle tone changes, which modulate retrograde flow and vascular resistance. Arm repositioning alters vascular filling, modifies arterial tone, and thereby adjusts retrograde flow magnitude and dicrotic curvature. The venulo-arteriolar reflex affects pulse wave amplitude but not dicrotic curvature, which is governed solely by arteriolar smooth muscle tone.

It should be noted that these conclusions are based on the results of a study of a group of young participants. In elderly patients, dicrotic curvature patterns may differ due to arteriolar wall stiffness and vascular pathologies (e.g., atherosclerosis, diabetes) [40].

Thus, both the amplitude and the morphology of the pulse wave on the dicrotic segment are essential components of photoplethysmogram analysis for diagnosing arterial vascular health and assessing changes in arterial resistance. When analyzing the pulse wave morphology on the diastolic segment, the arm's position relative to heart level must be accounted for.

Disclosures

All authors declare that there is no conflict of interest in this paper.

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An Exponential Model of a Three-Layered Medium Mimics Astrocytoma in Visible and Near-Infrared Ranges

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Abstract. Development of an exponential model of diffuse reflectance of three-layered media has been accomplished over visible and near-infrared ranges of wavelength. The optical properties of these layers were selected to mimic astrocytoma, grey matter, and white matter respectively. The medium was considered to be homogenous and mismatched boundaries were not taken into account. Then, the refractive index of these three layers was the same and equaled 1.4. The thickness of the tumor layer turned out to be varied between 100 μm and 1 cm and from 0.1 cm to 0.5 cm for the grey matter. The lowest layer, which mimics white matter, was considered to be a semi-infinite medium. Reflectance was estimated theoretically based on the Monte-Carlo algorithm and fitted to the developed model. Finally, the relative error of the presented model was varied from 10% at 450 nm to 1% at 1064 nm. The simplicity and robustness of the presented model make it a good choice for estimating reflectance and reproducing the thickness of the upper layer in real-time.

Keywords: Diffuse Reflectance; Astrocytoma; Monte Carlo; Brain Tissue.

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1 Introduction

Astrocytoma is a type of cancer that can occur in the brain and spinal cord, it begins in specific sorts of cells called astrocytes that support nerve cells and its symptoms based on the tumor's location. Widely known treatment of this sort of tumor is surgery during which the surgeon works to remove as much of the astrocytoma as possible. Like any type of cancer, the recurrence rate of astrocytoma depends mainly on the completeness of tumor resection, then, the observation of tumor margins is considered to be a potential task during surgery besides each surgeon needs an assistant tool to determine the tumor location in real time [1–4].

On the other hand, optical methods' use is widely spread in medicine and diagnosis as a non-invasive real-time approach [5]. One of these optical approaches is diffuse reflectance spectroscopy (DRS), which depends on the proportionality of physiological and optical properties of media to distinguish normal and tumor tissues [6]. To predict the relationship of diffuse reflectance spectra to optical properties, analytical and numerical solutions of the radiative transport equation have been developed [7]. Numerical solutions are

regarded as the most accurate methods even though they require more time to estimate the diffuse reflectance and vice versa [7]. Analytical solutions, on the other hand, are distinguished by their fast results with low robustness, which makes them more likely to be used in real time [7]. In the literature, there are many analytical models of diffuse reflectance of semi-infinite and two-layered tissues; most of them confirm the applicability to describe precisely light-tissue interaction [8–17]. Unfortunately, a few researchers have studied the diffuse reflectance spectrum of three-layered media [14–17]. In that context, Yudovsky and Durkin developed a hybrid model of skin tissue that combines two-flux approximation and diffusion approximation equation [14]. Some presumptions were considered during deriving a model, and the standard error of the presented model did not exceed 10% [14]. Furthermore, the three-layered exponential model was developed to identify the structural properties of leishmaniasis by comparing healthy skin tissue using diffuse reflectance measurements in which the relative deviation exceeded 10% regarding diffuse reflectance spectra [15]. However, the p -value turned out to be varied between 0 and 1 for the upper layer thickness of the skin model [15]. Another

research group utilized machine learning using the diffuse reflectance measurements to detect pigments of human skin. The robustness of this approach varied depending on the parameters that were predicted, which was 30% for water content [16]. Based on the same approach using neural network F-ANN and Monte-Carlo simulation, the water content and thickness of skin sub-layers could be estimated with minimal relative error approximated at 4% using spatially-resolved diffuse reflectance spectra [17]. To the best of our knowledge, there is no diffuse reflectance model for three-layered media that mimics the structural and optical properties of astrocytoma, grey and white matters of the human brain respectively, which might be used to predict the thickness of the astrocytoma and quantify other optical parameters.

The current work aims to develop a simple exponential model of diffuse reflectance for three-layered media mimicking the optical properties of astrocytoma, grey and white matter respectively. For these purposes, Monte Carlo simulation has been implemented to predict the diffuse reflectance of these media and the dataset has been fitted to the exponential model to predict the fitting parameters for all situations. Then, the robustness of this model was evaluated.

2 Materials and Methods

2.1 Exponential Model

Analytical approximation of the radiative transport equation is preferable, especially the exponential solution that supposes an exponential relationship of diffuse reflectance to absorption μ_a and reduced scattering μ'_s coefficients. For a tiny homogenous slab dz , diffuse reflectance can be expressed as following [9, 10]:

$$dR_d = 2\mu'_s \cdot e^{-2(\mu'_s + \mu_a)z} dz. \quad (1)$$

To estimate the total diffuse reflectance of a three-layered medium with varied optical properties and thicknesses of the superficial slabs, the expression could be presented by [9, 10]:

$$dR_t = dR_1 + dR_2 + dR_3 = \begin{cases} dR_1 = 2\mu'_s{}^{(1)} \int_0^{L_1} e^{-2(\mu'_s{}^{(1)} + \mu_a^{(1)})z} dz \\ dR_2 = 2\mu'_s{}^{(2)} \int_{L_1}^{L_2+L_1} e^{-2(\mu'_s{}^{(2)} + \mu_a^{(2)})z} dz \\ dR_3 = 2\mu'_s{}^{(3)} \int_{L_2+L_1}^{\infty} e^{-2(\mu'_s{}^{(3)} + \mu_a^{(3)})z} dz \end{cases} \quad (2)$$

Eq. (2) describes the diffuse reflectance of a medium with three layers, which is composed of the contribution of each layer. The absorption and reduced scattering coefficients ($\mu_a^{(i)}$ & $\mu'_s{}^{(i)}$ and $i = 1, 2, 3$). The thickness of the first and second layers that mimic the tumor and grey slab are L_1 and L_2 respectively. The third layer that mimics the white matter was considered a semi-infinite slab. By integrating, the

total diffuse reflectance might be presented as follows [9, 10]:

$$R_t = R_1 + R_2 + R_3 = \begin{cases} R_1 = \left(\frac{\mu'_s{}^{(1)}}{\mu'_s{}^{(1)} + \mu_a^{(1)}} \right) \times \\ R_2 = \left(\frac{\mu'_s{}^{(2)}}{\mu'_s{}^{(2)} + \mu_a^{(2)}} \right) \times \\ R_3 = \left(\frac{\mu'_s{}^{(3)}}{\mu'_s{}^{(3)} + \mu_a^{(3)}} \right) \times \end{cases} \times \left(1 - e^{-(\mu'_s{}^{(1)} + \mu_a^{(1)})L_1} \right) \times \left(e^{-(\mu'_s{}^{(2)} + \mu_a^{(2)})L_1} - e^{-(\mu'_s{}^{(2)} + \mu_a^{(2)})(L_1+L_2)} \right) \times \left(e^{-(\mu'_s{}^{(3)} + \mu_a^{(3)})(L_1+L_2)} \right). \quad (3)$$

Since the diffuse reflectance predicted by the exponential model does not match the measured reflectance, rescaling factors are introduced to satisfy scale invariance and reduce the relative errors. These semi-empirical parameters rely on the optical properties and the experimental setup characteristics that used to measure the diffuse reflectance [9, 10]. Then, the diffuse reflectance could be expressed as follows [9, 10]:

$$R_t = \begin{cases} R_1 = k_1 \cdot \left(\frac{\mu'_s{}^{(1)}}{\mu'_s{}^{(1)} + \mu_a^{(1)}} \right) \times \\ R_2 = k_3 \cdot \left(\frac{\mu'_s{}^{(2)}}{\mu'_s{}^{(2)} + \mu_a^{(2)}} \right) \times \\ R_3 = k_6 \cdot \left(\frac{\mu'_s{}^{(3)}}{\mu'_s{}^{(3)} + \mu_a^{(3)}} \right) \times \end{cases} \times \left(1 - e^{-k_2 \cdot (\mu'_s{}^{(1)} + \mu_a^{(1)})L_1} \right) \times \left(e^{-k_4 \cdot (\mu'_s{}^{(2)} + \mu_a^{(2)})L_1} - e^{-k_5 \cdot (\mu'_s{}^{(2)} + \mu_a^{(2)})(L_1+L_2)} \right) \times \left(e^{-k_7 \cdot (\mu'_s{}^{(3)} + \mu_a^{(3)})(L_1+L_2)} \right). \quad (4)$$

Furthermore, the amount of light reaching a given layer depends mainly on the optical penetration depth and the thickness of the superficial layer as discussed in our previous work [9]. When the effective attenuation coefficient of the first layer is high, the optical penetration depth of the first layer is low. Then, that minimizes the amount of light penetrates the second layer, then, the contribution of the second layer decreases. When the effective attenuation coefficients of the first and second layers are high and the optical penetration depths of these layers are low, the amount of light reaching the third layer is very low, which minimizes the contribution of the third layer in total diffuse reflectance. On the other hand, when the effective attenuation coefficient of the first layer is low, the optical penetration depth of the first layer is high. Then, that maximizes the amount of light penetrates the second layer and, then, the contribution of the second layer is increased. Furthermore, when the effective attenuation

coefficients of the first and second layers are low and the optical penetration depths of these layers are high, the amount of light can reach the third layer is very high, which maximizes the contribution of the third layer in total diffuse reflectance. Then, a modification of Eq. (3) might be applied using a hyperbolic tangent of the optical penetration depth that is the inverse of effective attenuation coefficient and the diffuse reflectance of the given medium with three layers could be expressed as follows [9]:

$$R_t = \begin{cases} R_1 = k_1 \cdot \left(\frac{\mu_s'^{(1)}}{\mu_s'^{(1)} + \mu_a^{(1)}} \right) \\ R_2 = k_3 \cdot \tanh \left(\frac{1}{\left(3 * \mu_a^{(1)} * (\mu_a^{(1)} + \mu_s'^{(1)}) \right)^{\frac{1}{2}}} \right) \\ R_3 = k_6 \times \tanh \left(\frac{1}{\left(3\mu_a^{(1)} (\mu_a^{(1)} + \mu_s'^{(1)}) \right)^{\frac{1}{2}} \left(3 * \mu_a^{(2)} * (\mu_a^{(2)} + \mu_s'^{(2)}) \right)^{\frac{1}{2}}} \right) \end{cases} \quad (5)$$

$$\begin{aligned} & \times \left(1 - e^{-k_2 \cdot (\mu_s'^{(1)} + \mu_a^{(1)}) L_1} \right) \\ & \times \left(\frac{\mu_s'^{(2)}}{\mu_s'^{(2)} + \mu_a^{(2)}} \right) \left(e^{-k_4 \cdot (\mu_s'^{(2)} + \mu_a^{(2)}) L_1} - e^{-k_5 \cdot (\mu_s'^{(2)} + \mu_a^{(2)}) \cdot (L_1 + L_2)} \right) \\ & \times \left(\frac{\mu_s'^{(3)}}{\mu_s'^{(3)} + \mu_a^{(3)}} \right) \left(e^{-k_7 \cdot (\mu_s'^{(3)} + \mu_a^{(3)}) (L_1 + L_2)} \right) \end{aligned}$$

2.2 Monte Carlo Simulation

The Monte-Carlo algorithm can solve the radiative transport equation (RTE) accurately for all cases and experiments, which makes it more likely to be a standard method [18, 19]. The Monte-Carlo method depends on the random sampling of variables from a probability distribution. For a random variable, there is a probability density function that defines the distribution of random variables over a specific interval. This random variable may be the variable step size between two interaction sites or the angle of deflection taken by a photon due to a scattering event. The computer generates a random variable that used to estimate the step size, and position of a photon packet with an initial weight is equal to 1. The optical properties of the medium describe the probability of photons to be absorbed or scattered per unit

pathlength. The weight of the photon is calculated after each event, and a new position is determined and so on until the weight of the packet is less than a specific value then the photon packet is terminated and a new photon packet is introduced and so on until all packets are terminated. The distribution of absorbed and scattered energy can be calculated and plotted in the Cartesian coordinates. The Monte-Carlo of Multi-Layers (MCML) code was used in the present study to predict the total diffuse reflectance of a three-layered medium. That simulates light propagation of infinitely narrow beam that incidents perpendicularly upon a surface of a multi-layered medium with distinct optical properties and the same refractive index. The medium is structured with three layers to mimic the brain structure with optical properties matching the optical characteristics of astrocytoma, grey matter, and white matter respectively, Table 1 [20, 21]. The superficial layer thickness turned out to be varied between 100 μm and 1 cm, and the grey thickness varied from 0.1 cm to 0.5 cm. The lower layer mimicked optically the white matter, which was assumed to be a semi-infinite medium with a thickness equaling 10 cm. The refractive index of these layers was chosen to be identical and equaling 1.4. MCML code used in the present study was supplied by a Matlab interface, which was implemented for 0.5 million photons incident perpendicularly on the medium [18, 19]. In order to implement MCML code, a layer matrix of three layers should be introduced, which contains refractive index, absorption coefficient, scattering coefficient, anisotropy factor, and thickness respectively of each layer. By using the MCML function, Monte-Carlo simulation will start and then the results will be shown in Matlab [19].

3 Results

Depending on the MCML code, the total diffuse reflectance of three-layered media was estimated which mimic the optical properties of astrocytoma, grey matter, and white matter respectively. Fig. 1 represents the relation of total diffuse reflectance to top layer thickness (astrocytoma) for different grey matter thicknesses in visible and near-infrared ranges of wavelength. It is shown that the diffuse reflectance decreases exponentially as the top layer thickness increases. At a definite wavelength, the diffuse reflectance is inversely proportional to a top layer thickness. Furthermore, reflectance decreases as the second layer increases at the same top layer thickness as shown in Fig. 1.

Table 1 Depicts the optical properties used in our study [20, 21].

	Astrocytoma				Grey Matter				White Matter			
Wavelength, nm	450	510	670	1064	450	510	670	1064	450	510	670	1064
μ_a , cm^{-1}	3	2	0.5	0.6	0.7	0.4	0.2	0.5	1.4	1	0.7	1
μ_s , cm^{-1}	180	150	120	90	117	106	84	57	420	426	401	296
g	0.9	0.95	0.95	0.96	0.88	0.88	0.9	0.9	0.78	0.81	0.85	0.89

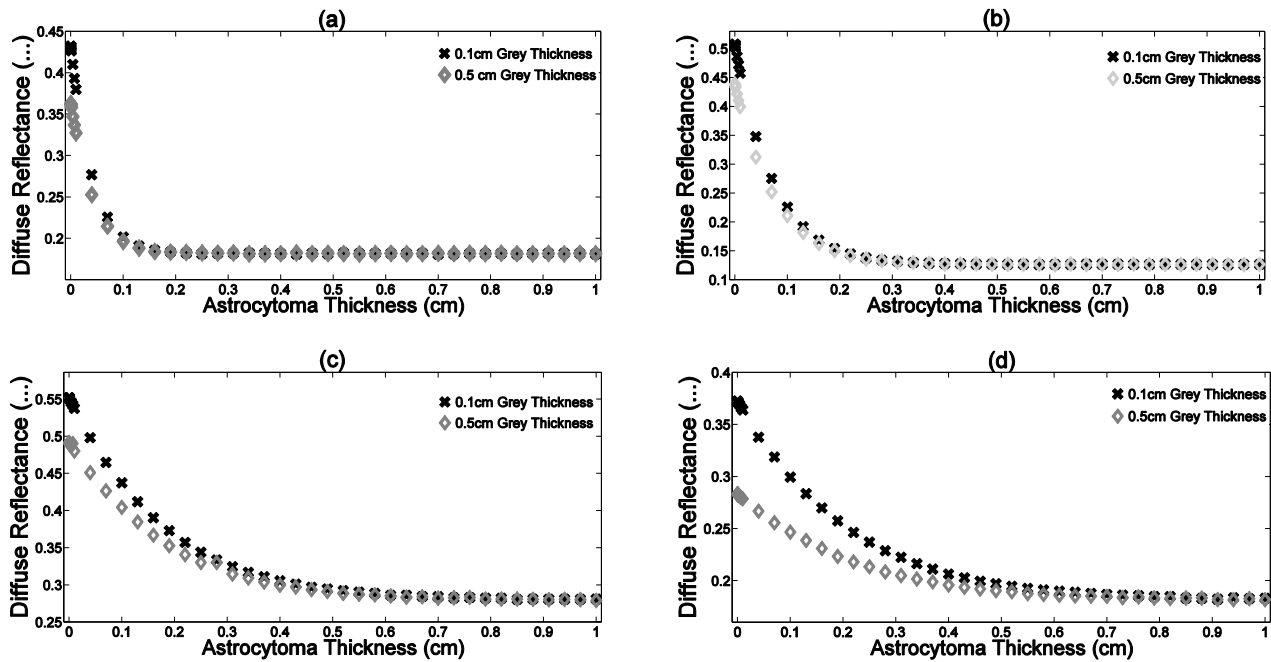


Fig. 1 Depicts the correlation of diffuse reflectance estimated by Monte-Carlo simulation at (a) 450 nm, (b) 510 nm, (c) 670 nm and (d) 1064 nm for different grey thickness.

Table 2 Depicts the fitting parameters of Eq. (5) for different thicknesses of the top layer.

Fitting Coefficient	k_1	k_2	k_3	k_4	k_5	k_6	k_7
450 nm	0.211947	0.810637	5.613341	1.383117	6.692463	-1281.25	1.845057
510 nm	1.59667	0.928607	3.492444	0.865016	1323.136	37175.62	1.196813
670 nm	0.305519	0.5574	1.596424	0.508423	1.045791	4.160872	0.127766
1064 nm	0.214348	0.822458	0.851312	0.615207	1.382076	3.710229	0.204534

Monte-Carlo results were fitted to the exponential equation developed for three-layered homogenous media (Eq. (5)) using a fitting function procedure in Matlab (Matlab 2015, Mathworks Inc, USA). The fitting coefficients were predicted for the media at each wavelength and depicted in the following table, Table 2.

Then, the diffuse reflectance was calculated by the fitted equation (Eq. (5)), and compared to the Monte-Carlo results, the relative standard error was calculated by the following Eq. (6):

$$\text{Relative Standard Error} = \left(\frac{R_{MC} - R_{predicted}}{R_{MC}} \right) * 100\% . \quad (6)$$

Fig. 2 shows a comparison of diffuse reflectance calculated by Monte-Carlo and Eq. (5) for each wavelength. The convergence was obvious and the relative error for our developed model was less than 10% for 450 nm, as shown in Fig. 2. For 510 nm, the relative error did not exceed 7% for all situations and 2% with at 670 nm. The relative error for diffuse reflectance predicted by Eq. (5) in comparison to the Monte-Carlo simulation did not exceed 1% for all thicknesses of grey and astrocytoma layers with one case with 33% relative error at 1064 nm.

4 Discussion

Diffuse reflectance of three-layered media, which mimicked the optical properties of astrocytoma, grey matter, and white matter respectively, was estimated using the Monte-Carlo simulation over visible and near-infrared range of wavelengths, Table 1. Then, a developed exponential model was fitted to the Monte-Carlo results to estimate the fitting coefficient that proposed for scale invariance to reduce the divergence between both methods, Table 2. The thicknesses of both the first and second layers were varied to meet the true biological regions. The results of the developed model (Eq. (5)) and the Monte-Carlo were compared to one another. Generally, the developed model was sensitive to the variation of thickness of the first and second layers, Fig. 2. Furthermore, the results of Eq. (5) were compatible with the Monte-Carlo simulation in spite of the fact that some situations with high relative error. Generally, the relative error of the developed model does not exceed 10% at 450 nm, 7% at 510 nm, 2% at 670 nm, and 1% at 1064 nm, which makes it more likely to use as an alternative method of Monte-Carlo simulation in real-time for the predefined medium.

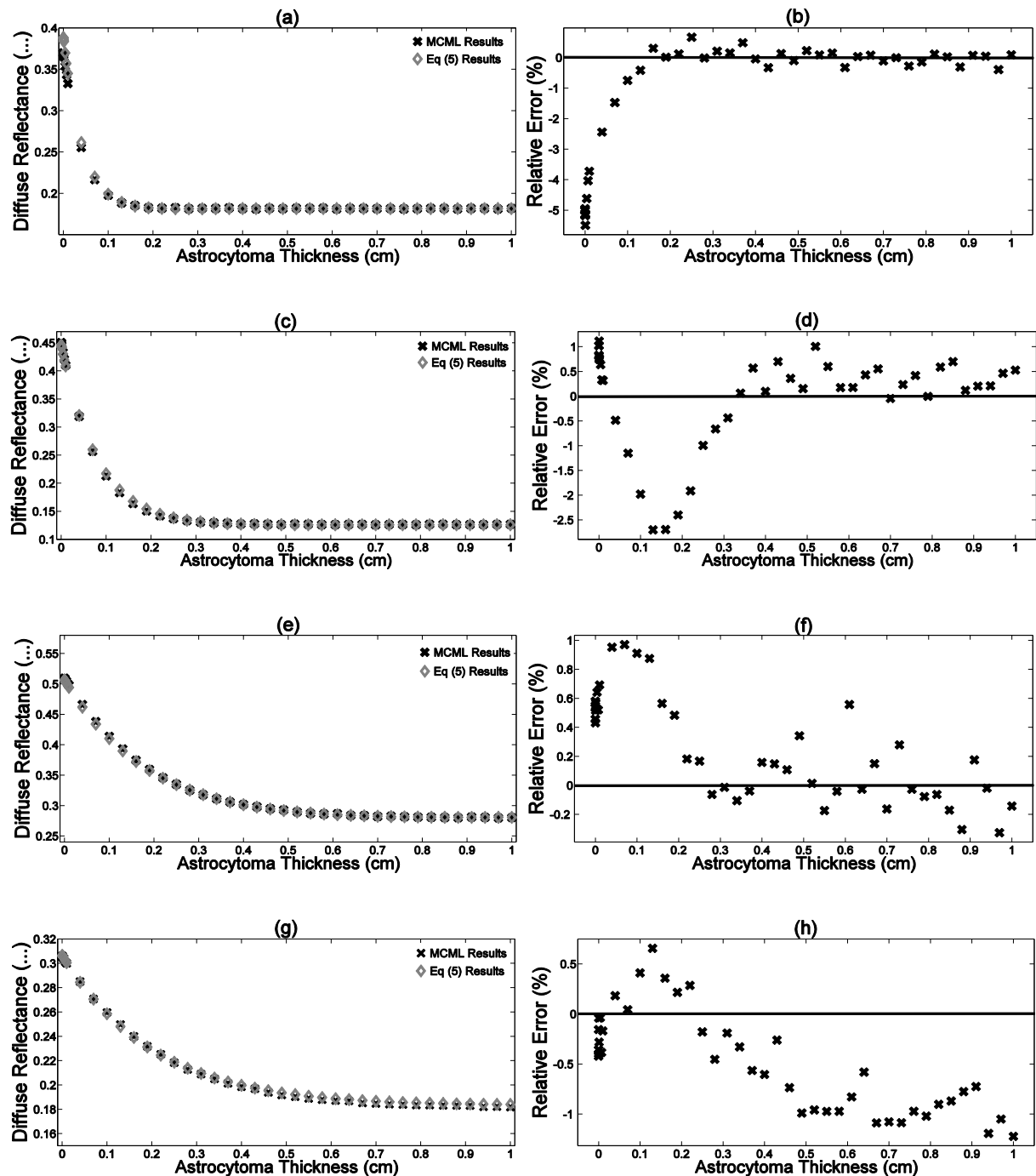


Fig. 2 Comparing of diffuse reflectance estimated by Monte-Carlo simulation (black) and Eq. (5) (grey) at (a) 450 nm, (c) 510 nm, (e) 670 nm and (g) 1064 nm respectively and the correlation of standard error of diffuse reflectance calculated at (b) 450 nm, (d) 510 nm, (f) 670 nm and (h) 1064 nm at 0.3 cm grey thickness.

The high error of Eq. (5) at the shorter wavelengths results from highly scattering properties of mimicked tissue at 450 nm and 510 nm in comparison to the others [14]. However, the developed model's error was less than the error of the three-layered exponential model used by other research groups due to the hyperbolic tangent (Eq. (5)) being introduced in our model [15]. That term makes a balance of contributing of the lower layers in the total diffuse reflectance of the medium. Furthermore, the relative standard error of our

developed model was comparable to other models developed by other research groups even though it is considered to be the simplest with valuable results in comparison to the others [14–17].

In summary, the present work was an attempt to investigate the exponential model whether it is possible to use it to model diffuse reflectance spectra of multi-layered medium. A valuable result of this developed model and low relative error make it more likely to predict precisely the diffuse reflectance and reproduce

the thickness of the tumor layer's thickness if the diffuse reflectance is measured. The weak point of this expression is that it simulates total diffuse reflectance, which could not be collected by optical fibers and requires an integrating sphere to be collected.

5 Conclusion

In the present work, an exponential model of diffuse reflectance was developed to mimic a three-layered medium with optical properties corresponding to astrocytoma, grey matter, and white matter respectively. Monte-Carlo simulation was used to generate a data set of our work and the developed model was fitted to these results.

Then, the model results were compared to Monte-Carlo, which considered a standard method to solve the radiative transport equation, the relative standard error ranged between 10% and 1% as wavelength increases. That is explained by the high scattering property of tissue in the visible range of wavelength. The relative error of

our presented model was compatible with other complicated model developed by the others. This convergence and simplicity of our model make it more likely to use and reproduce the thickness of the tumor, which could help surgeons to determine the structural properties of the tumor in real-time.

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Conflict of Interest

The author has no conflict of interests

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Capture and Retention of Polystyrene Microspheres with Biomarkers in Optical and Optothermal Traps

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Abstract. The capabilities of optical and optothermal tweezers for manipulating 2- μm polystyrene microspheres with kidney and thyroid antigens are experimentally demonstrated. The ability of arranging such objects in various configurations with subsequent fixation has been demonstrated. It has been shown that the detachment rates of modified polystyrene microspheres depend on the presence and type of antigen. Experiments on optical capture of polystyrene microspheres in a mixed solution of sodium tetraborate with the addition of sodium dodecyl sulfate (SDS) of different molar concentrations are carried out. It is shown that an increase in the molar concentration of SDS from 5 to 30 mM leads to a decrease in the detachment rate by 55%.

Keywords: optical tweezers; optothermal trap; laser manipulation; polymer microspheres; antigens; mixed solutions.

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1 Introduction

An optical trap (optical tweezers) is a method based on the use of the light power of a tightly focused laser beam to capture, hold and manipulate micro- and nanoscale objects [1–3]. In optothermal traps, a focused light field is used not only to generate optical power, but also to create a temperature gradient in the medium by absorbing laser radiation and, accordingly, convection currents that carry particles to the region of maximum radiation intensity [4–7]. Structured light fields significantly expand the functionality of both optical [2, 8–14] and optothermal [7, 15–19] traps. The methods are widely used in scientific research, as they allow precise manipulation of objects without physical contact, which minimizes the risk of damage. Micro- and nanoparticles of various natures, sizes, and morphologies are used as objects of manipulation: polymer, silicon, metal, microdroplets of organic solutions [20], as well as erythrocytes, leukocytes, DNA molecules, proteins, single cells, bacteria, etc. [3, 21–23]. Since the efficiency of capture depends significantly on the size of the objects, their shape, optical and physical-chemical properties, the parameters of the trap (power and wavelength of laser radiation, distributions of intensity and/or phase of the

light field, topological charge of the trap) are selected for each specific case. For example, the possibility of capturing is determined by the ratio between the refractive indices of the micro-object and the surrounding medium; to capture absorbing objects, it is necessary to create light traps with a special shape, etc. Optothermal traps are less sensitive to the characteristics of the object, but even in this case, the dynamic properties of the micro-object in the trap (and, accordingly, the efficiency of capture) are determined by its characteristics and require separate investigation.

Polymer microspheres modified with biomarkers (antigens, proteins, etc.) are of great practical interest as a basis for test systems for the rapid and effective diagnosis of various diseases, detection of infections and viruses, and they are also used in various analysis methods and as biosensors [24–27]. Considering the wide range of applications for diagnostic and biotechnological tasks, both fundamental and applied, it is interesting to explore the capabilities of laser manipulation of the microobjects under consideration. Microspheres labeled and unlabeled with biomarkers are of interest from the point of view of optical manipulation as objects of the same size, practically indistinguishable

externally, but with different physicochemical properties, as objects for sorting.

The aim of this article was to study the capabilities of an optical and optothermal trap for manipulating polystyrene microspheres with kidney and thyroid antigens. It was important to demonstrate the operability of schemes of an optical trap and our previously proposed optothermal traps [7, 16, 17] for these types of objects, as well as to test the sensitivity of optical tweezers to the objects under consideration i.e. to test the ability to distinguish between unmarked and marked with different antigens microspheres using the optical tweezers technique. The sensitivity of the optical tweezers was assessed by measuring the rate of detachment of the microsphere from the trap – the maximum rate of retention of the captured object in the trap. The parameter allows one to estimate the rigidity of a particular trap for capturing a specific object under given condition, and it is quite easy to measure. For the case of known characteristics of the surrounding solution (distilled water), the optical trapping forces were calculated. The detachment rates of polystyrene microspheres in a mixed solution of sodium tetraborate with the addition of sodium dodecyl sulfate (SDS) of different molar concentrations were also measured.

2 Materials and Methods

The micromanipulation samples were polystyrene microspheres (Alpha Nanotech Inc.) of 2 μm size, pure and with thyroid antigens or kidney antigens. The finished samples – microspheres with kidney antigens or thyroid antigens mixed with distilled water – were provided by the Analytical Research and Development Laboratory of Reaviz Medical University in sealed Eppendorf-type test tubes. To conduct experiments on optical manipulation, the sample tube was shaken to evenly distribute the microspheres in the liquid volume; 1 μl of the sample suspension, taken from the middle of the tube using a Thermo Fisher Scientific single-channel

laboratory pipette, was mixed with 1 ml of distilled water and thoroughly mixed. Physiological saline (0.9% NaCl), physiological saline with sodium dodecyl sulfate, sodium tetraborate buffer solution ($\text{Na}_2\text{B}_4\text{O}_7$) and SDS solutions in sodium tetraborate with different molar concentrations of 5–30 mM were also used as solutions.

The experiments were carried out using optical and optothermal trap techniques. A typical scheme of a structured optothermal trap is shown in Fig. 1. In the experiments, a diode-pumped solid-state laser (DPSS) was used. The radiation wavelength was 0.53 μm . The output power varied from 10 to 60 mW. The structured trap (a trap with a complex intensity distribution – in the form of a ring or a square contour) was formed by using a liquid crystal spatial light modulator (LC SLM) HOLOEYE PLUTO-2-NIR-011 (1080×1920 px, pixel size 8 μm , 256 grayscale, modulation depth 2π), operating in reflection mode. Phase masks supplied to the LC SLM were calculated using spiral beam optics [28]. An expanded collimated beam illuminated the LC SLM, and the formed structured beam was focused by a 90× microscope objective. Microparticles suspended in solutions cylindrical cuvette (diameter 30 mm, thickness 40 mm, liquid layer thickness – 1–1.5 mm) with an absorbing bottom: an orange OC 13 filter was used as the bottom. Due to the absorption of radiation with a wavelength of 0.53 μm , a temperature gradient and, accordingly, a convection flow arose.

The same circuit elements were used to form a point optical trap. The SLM could be switched off and used as a mirror. In our experiments, the phase distribution of the lens was set to the modulator and focusing was done through it. For experiments with optical tweezers, we used a cell with a transparent bottom (K8 glass).

In experiments on capturing and aligning up microspheres with antigens into a complex contour we have added albumin to the cell and increased the laser power to fix the formed structures. We have proposed and described a technique for fixing microobjects along the boundaries of a structured trap in Ref. [17].

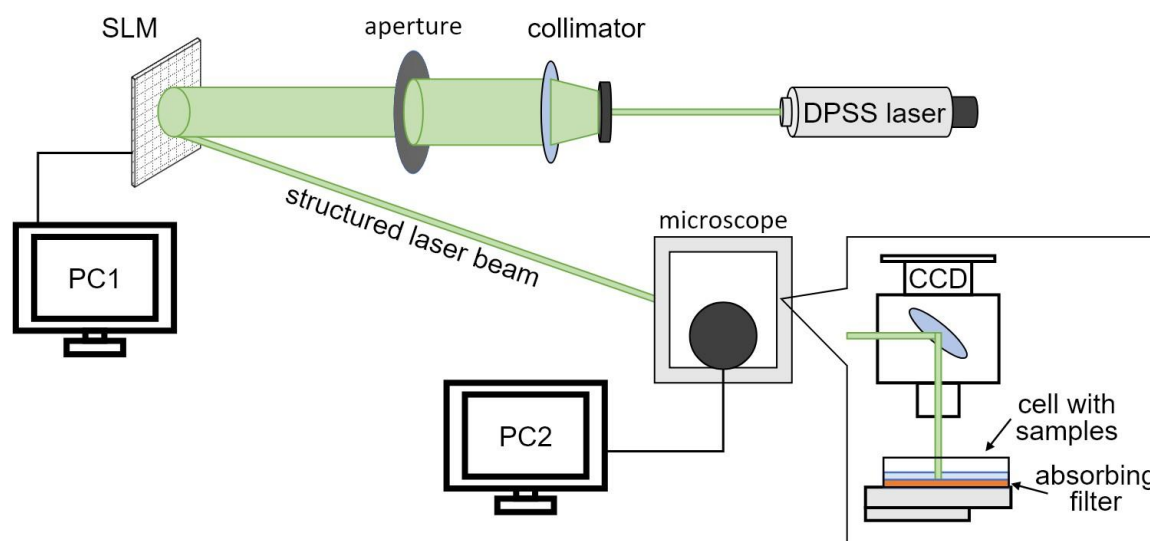


Fig. 1 The scheme of structured optothermal trap.

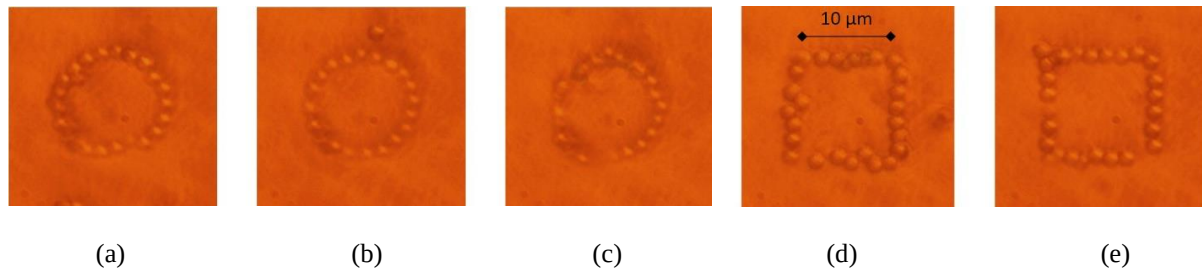


Fig. 2 Results of experiments on optothermal fixation of 2 μm latex microspheres with kidney cells immobilized on the surface in the form of a ring (a–c) and a square (d–e) due to albumin coagulation at a radiation power of 60 mW. In all cases (a–e), a uniform distribution with small differences can be observed.

3 Experiments and Discussion of Results

3.1 Experiments with an Optothermal Trap: Capture, Alignment along the Trap Boundary, and Subsequent Fixation

In experiments with an optothermal trap, the transfer of microparticles with kidney antigens by convection flows and their alignment along the trap boundary was recorded. The radiation power for capturing and moving microparticles was 20–30 mW. A 2.5% albumin solution was used for fixation. The particles actively moved towards the trap from all distances within the microscope's field of view and aligned fairly evenly along the ring or square's border. Fixation of microobjects on the substrate was achieved by a short-term increase in laser power to ~ 60 mW, which led to coagulation of albumin. Fig. 2 shows examples of frames from a video of microsphere fixation. It is evident that in all cases it is possible to evenly arrange and fix the microspheres along the trap boundary.

Microparticles with thyroid antigens could not be aligned along the trap boundary. This was due to the very slow movement of microparticles at laser powers of 10–30 mW. Increasing the power to 60 mW did not result in active particle movement. Further increasing the power under the given experimental conditions was not possible due to albumin coagulation.

In Refs. [16, 17], we have demonstrated the capture and alignment of latex microspheres of various sizes (2 and 4 μm) along a ring with a total laser radiation power in the working zone $27 \div 100$ mW and a $40\times$ magnification of the microobjective. In Refs. [7, 16], the radial and vertical components of the convection flow in point and ring optothermal traps were modeled depending on the laser radiation power. The results of the simulation, confirmed by the results of the experiments, showed that when working with an optothermal trap, it is crucial to adjust the power level of the laser light in a manner that ensures the particles are captured by the trap, but not carried upwards by the rising convective currents of the fluid. Although the ring-shaped optothermal trap is characterized by a less sharp increase in the vertical component of the flow velocity as it approaches the illuminated region compared to the point-shaped trap, this dependency is quite significant for latex particles. Therefore, the virtual absence of influence of laser

radiation power on the behavior of microparticles with thyroid antigens most likely indicates a change in their adhesive properties.

3.2 Experiments with a Point Optical Trap: Determination of the Detachment Rates of Microspheres

Experiments were conducted to study the capture and motion of polystyrene microspheres and polystyrene particles with antigens using an optical trap. We determined the maximum velocities of movement (detachment rates) of these particles. The breakaway force method was used to estimate the maximum speed of motion. A cell with a drop of each suspension was mixed with 1 ml of distilled water and placed on the microscope stage. A separate microsphere was captured by a point optical trap (a focused beam of laser radiation) and moved in a straight line. The speed of movement was increased until the microparticle escaped out of the beam. Experiments were repeated at least 5 times with each microparticle, using at least 10 microspheres in each series.

The release of microparticles occurs at the moment when the capture force, which is dependent on the power of the laser radiation, light beam parameters and the size and relative refractive index of the microobject, equals the viscous friction force determined by the Stokes Eq. (1):

$$F_{drag} = 6\pi\eta rV, \quad (1)$$

where r is the radius of the microsphere, η is the viscosity coefficient, V is the velocity. Thus, the optical trapping force was calculated using the Eq. (1), where the viscosity coefficient η for distilled water was equaled to 0.001 Pa $\times$ s (at a temperature of 20 $^{\circ}\text{C}$). The calculation results are presented in Table 1 and, for clarity, in Fig. 3.

As can be seen from the results, application of antigens leads to a change in detachment rate. At that, detachment rates and forces of optical trap for polystyrene with thyroid antigens are significantly lower than those for polystyrene microspheres or for polystyrene with kidney antigens. This is consistent with the results of optothermal trap experiments and is apparently associated with change in adhesive properties of microparticles upon application of different types of antigens.

Table 1 Experimental values of the detachment rate and strength of the optical trap.

Microobjects under study	Solution	Detachment rate, $\mu\text{m/s}$	Optical trapping force, fN
2 μm polystyrene microspheres	distilled water	39.4 ± 6.3	742.2 ± 133.6
2 μm polystyrene microspheres with thyroid biomarkers	distilled water	20.3 ± 5.5	382.0 ± 101.4
2 μm polystyrene microspheres with kidney antigens	distilled water	44.9 ± 9.9	845.8 ± 192.3
2 μm polystyrene microspheres	saline solution (0,9% NaCl)	31.0 ± 4.2	—
2 μm polystyrene microspheres	saline solution +5 mM SDS	21.0 ± 3.5	—

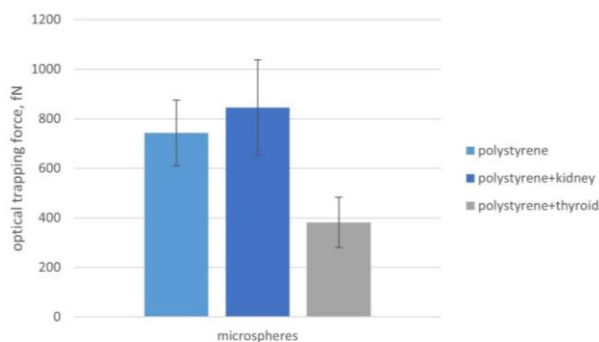
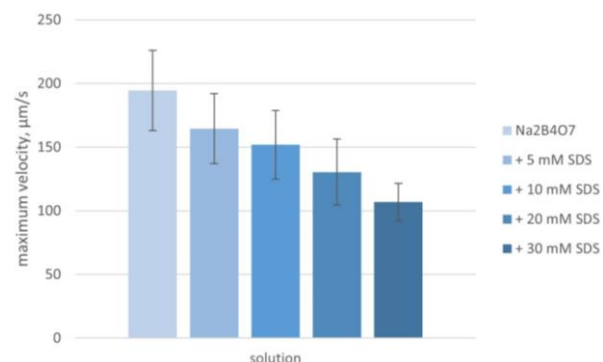
Fig. 3 Dependences of optical trapping forces for different types of 2- μm microspheres in distilled water at a laser radiation power of ~ 20 mW.

Table 1 also shows the values of the detachment rate of polystyrene microparticles suspended in saline and saline with the addition of SDS. A decrease in the detachment rate is observed. This is explained by a slight increase in the refractive index of saline compared to the refractive index of water, i.e. a decrease in the relative refractive index of polystyrene in saline, as well as an increase in the viscosity of saline. Thus, the optical trapping force, which is directly proportional to the relative refractive index of the particle in the medium, decreases, and the viscous friction force increases. The addition of sodium dodecyl sulfate further increases the viscosity of the solution, which leads to a decrease in the detachment rate. Since the exact values of the viscosity coefficient for the solutions used are unknown, the optical trapping force was not calculated.

From the point of view of biotechnological applications of optical tweezers, manipulation in various buffer solutions, including those with the addition of organic compounds, is of particular interest. Experiments were carried out to measure the detachment rate of 2- μm polystyrene particles (without biomarkers) in SDS solutions in $\text{Na}_2\text{B}_4\text{O}_7$ of different molar concentrations. In this series of experiments, we increased the power to 60 mW, as this allows for increased speed and accuracy of micro-object capture without the risk of damaging the biomarker. The results are presented in Table 2 and in Fig. 4.

Table 2 Experimental detachment rates of 2- μm polystyrene microspheres in $\text{Na}_2\text{B}_4\text{O}_7$ solutions with the addition of SDS of different concentrations.

Solutions under study	Detachment rate, $\mu\text{m/s}$
$\text{Na}_2\text{B}_4\text{O}_7$	194.5 ± 31.5
$\text{Na}_2\text{B}_4\text{O}_7$ + 5 mM SDS	164.7 ± 27.5
$\text{Na}_2\text{B}_4\text{O}_7$ + 10 mM SDS	151.9 ± 27.1
$\text{Na}_2\text{B}_4\text{O}_7$ + 20 mM SDS	130.4 ± 26.0
$\text{Na}_2\text{B}_4\text{O}_7$ + 30 mM SDS	106.9 ± 14.8

Fig. 4 The detachment rate of 2- μm polystyrene microspheres depending on the molar shell of SDS in $\text{Na}_2\text{B}_4\text{O}_7$. Laser radiation power was ~ 60 mW.

Increasing the power compared to previous experiments resulted in an increase in the detachment rate, which decreased with increasing SDS concentration. The detachment rate decreases by 55% with increasing SDS molar concentration from 5 to 30 mM. It is well known that sodium dodecyl sulfate is an organic compound with the formula $\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3\text{Na}$ and is an anionic surfactant. Increasing the concentration of surfactants resulted in the microparticles not sticking to the surface, but their dynamic properties deteriorated.

As articles review shows, the dynamic behavior of microparticles in optical traps in organic and mixed solutions is highly complex. Thus, the authors of Ref. [29] note the independence of the rigidity of the

optical trap from the refractive index of organic solvents when capturing polystyrene particles coated with a layer of fused quartz. The work shows that the rigidity of the trap, regardless of the refractive index, was about ~ 0.15 pN/nm for a large number of studied organic solvents (ethanol, isopropanol, acetone, DMF, and others), and the dynamics of microobjects in the trap was determined by the viscosity of the solution. In the work of T. Kužela et al. [30], a systematic study of the behavior of a micro-object in an optical trap depending on the concentration of a mixed solution is carried out for the first time. The authors investigate the dynamic behavior of polystyrene microparticles of $0.8\ \mu\text{m}$ and $3\ \mu\text{m}$ in mixed solvents, which include water with the addition of dimethyl sulfoxide, ethylene glycol and glycerol. It is shown that for all the solvents used, an increase in viscosity is observed with an increase in the molar concentration of organic compounds from 5 to 30 mM, which indirectly confirms the results of our measurements.

Experiments conducted with polystyrene microspheres showed that the type of solution and changes in the concentration of substances in the solution affect the interaction between the laser and the object, changing the characteristics of capture and retention. Studies of the dynamics of polymer microspheres under the influence of optical traps in various media are interesting from a fundamental point of view, because a change in concentration leads to a continuous change in the refractive index and viscosity of the solution. And they will be continued in the future.

4 Conclusion

Experiments were conducted with pure polystyrene microspheres and polystyrene microspheres with kidney and thyroid gland antigens using optical and optothermal trapping techniques. The ability of capturing and transferring polystyrene microspheres with kidney antigens from relatively large distances using an optothermal trap, aligning them into specific configurations formed by structured light traps, followed by fixation using an albumin solution and a short-term increase in power is demonstrated. In this work, traps in the form of a circle and a square boundary were used, but the light fields can be quite diverse.

It was shown that the speeds of detachment of microobjects from the optical trap are different for pure

polystyrene microspheres and polystyrene with biomarkers, which in the future can be used to sort microobjects of the same size, but with different chemical and physical properties. However, a large number of experiments with subsequent image processing are required to collect statistics and calibration.

The detachment rates of polystyrene microspheres (without antigens) in various solutions were measured. It was shown that this value depends on the choice of solution and the concentration of dissolved substances. In particular, in a mixed solution of sodium tetraborate, the detachment rates of polystyrene microspheres decrease by 55% with an increase in the molar concentration of SDS from 5 to 30 mM, which may be associated with an increase in the viscosity of the solution in this range of molar concentrations. Thus, our experiments confirmed the possibility of controlling the capture rate by choosing a solution and changing the quantitative composition of the mixed solvent. The polystyrene microspheres used at this stage can be considered as model objects with known optical properties. The presence of a biomarker (antigen) significantly changes the physicochemical properties of the microspheres and, accordingly, their movement in the optical trap. On the other hand, the refractive index of the solution, its viscosity, solubility and other parameters can significantly affect the motion of an object in it. In the future, studies will be conducted on the movement of modified microspheres with various antigens in practically significant buffer solutions. One example is a buffer system based on Tween 80, which is used in diagnostic test systems that employ the indirect agglutination reaction. Studies of the movement of polystyrene microspheres in mixed solutions will also be continued.

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Disclosures

The authors declare that they have no conflict of interest.

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Factors Determining the Increased Sensitivity of Cancer Cells to the Action of Optical Radiation in Blue Spectral Region

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Abstract. The majority of studies state that inactivating effect of blue light is more pronounced in relation to cancer cells. We show, for the first time, that one of the reasons for increased sensitivity of cancer cells to blue light is higher concentrations of endogenous porphyrin sensitizers in them, which is confirmed by fluorescence methods. This leads to higher levels of light-induced production of reactive oxygen species in cancer cells, detected by chemiluminescence, and higher rate of light-induced decrease in metabolic activity of cancer cells compared to normal cells. The decisive role of endogenous porphyrins is evidenced by higher rate of photoinactivation of cells and more intense chemiluminescent signal when cells are exposed to laser radiation with wavelength of $\lambda = 405$ nm, corresponding to the maximum of absorption spectrum of porphyrins, compared with $\lambda = 445$ nm, corresponding to the maximum of absorption spectrum of flavins.

Keywords: cancer cells; 405 nm; 445 nm; endogenous porphyrins; flavins; reactive oxygen species.

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1 Introduction

Currently, the ability of low-intensity (0.5–100 mW/cm² irradiance) optical radiation in blue spectral region ($\lambda = 400$ –485 nm) to influence the functional characteristics of cancer cells *in vitro* is beyond doubt [1–3]. There are numerous reports on the inhibitory effect of blue light in the literature on colon cancer cells [4–11], melanoma [2, 12–22], carcinoma [18, 23–33], sarcoma [3, 5, 34–37], lymphoid cells [38–41], leukemia cells [42–46], bladder cancer cells [47, 48], pancreatic [49] and mammary [30, 50, 51] gland cancer, hepatoma (liver cancer) cells [52, 53], gliomas [54], glioblastomas [55, 56], neuroblastomas [30, 57], stem cells of tumor tissue [9, 58], prostate cancer cells [59], lung cancer [60–62], cancer-associated fibroblasts [10] and others.

An analysis of previously performed studies allows us to conclude that exposure of cancer cells *in vitro* to blue light in the energy dose range of 1–250 J/cm² leads

to a dose-dependent decrease in their survival, suppression of proliferation, and a decrease in the ability to migrate and invade. Exposure to blue light causes apoptosis [4, 7, 8, 14, 19, 22, 23, 27, 30, 31, 36–39, 41, 43–45, 49, 52–54, 56], necrosis [16, 30, 56, 57], autophagy [4, 6, 10, 21, 35, 38, 41] and disruption (arrest) of the cell cycle [4, 19, 22, 27, 43, 47, 49, 54, 57]. The occurrence of these processes in cells is facilitated by increased production (under blue light) of reactive oxygen species (ROS) [7, 8, 16, 19–21, 23, 28, 30, 35, 36, 38, 41, 60] and DNA damage at relatively high energy doses [7, 20, 22, 31, 38, 41, 52, 63].

It is characteristic that in studies in which, *in vitro*, the effect of light of the same spectral and energy parameters on cancer and non-transformed cells was compared, the presence of pronounced differences in their response to the influence of the specified physical factor was noted [4, 8, 23, 28, 31, 33, 36, 39, 46, 48, 49, 57, 59, 60]. These differences are manifested in a higher degree of

photoinhibition of metabolic activity and cell proliferation [4, 8, 23, 31, 33, 39, 46, 48, 49, 57, 59, 60], as well as higher levels of light-induced ROS generation [23, 28, 60, 33, 39, 48]. Moreover, the difference in the reactions of cancer and non-transformed cells to light exposure is observed in almost the entire spectral range of blue light: 450 nm [31, 48], 456 nm [39], 460 nm [49], 465 nm [4, 8, 36], 460–470 nm [57], 470 nm [46], 473 nm [60], 485 nm [59], 400–500 nm [23, 28, 33], 450–500 nm [33] as when exposed to laser [33, 48, 60], and LED [4, 8, 31, 36, 39, 46, 49, 57] and lamp (broadband) [23, 28, 33, 59] sources.

However, the reasons for the increased sensitivity of cancer cells to the action of blue light remain unclear. In our opinion, a complicating factor in resolving this issue is the lack of complete understanding of the mechanisms of photophysical and photochemical processes that determine the effects of photobiomodulation initiated by exposure to blue light. The question of the primary acceptor molecules responsible for the regulatory action of this physical factor remains the least studied and most debated. At the same time, the opinion has been repeatedly expressed about the multiplicity of molecular targets capable of absorbing light quanta in the blue spectral region and mediating the reaction of animal cells to such exposure [1, 64–66]. Traditionally, the respiratory chain enzyme (electron transport chain) cytochrome c oxidase (CCO), as well as flavoproteins (“cryptochromes”) and nitrosated proteins are considered as main targets of blue light [1, 64–66]. In addition, evidence has been obtained of the important role of light-sensitive ion channels in the photoregulation of the metabolic activity of cells, the best known of which are channelrhodopsins, which absorb light in blue spectral region [1, 6, 54, 65–70]. Another family of broad group of light-sensitive cation channels are transient receptor potential channels (TRP) [1, 65, 71], which are found in most organisms, tissues, and cell types.

At the same time, animal cells also contain other chromophores that are potentially capable of acting as molecules-acceptors of optical radiation in various regions of the visible spectrum and initiating a change in the redox state of cells due to the generation of reactive oxygen species [72–81]. Thus, a number of studies argue for the participation of endogenous flavins [29, 78, 79] and lipofuscin [80, 81] in the effects of photobiomodulation in cells under *in vitro*. Our laboratory has obtained evidence of the participation of endogenous porphyrin photosensitizers in the implementation of the regulatory effect of light in various types of cells: microorganisms [73], sperm [74, 75], human blood cells [76], somatic cells [29, 77].

The purpose of this work is to substantiate the important role of endogenous porphyrins in the sensitization of photobiological processes in cancer and normal cells when their suspension is exposed to blue light and to clarify the role of these tetrapyrroles in the manifestation of increased sensitivity of cancer cells exposed to blue light.

2 Materials and Methods

2.1 Cell Culture

Human cervical epithelioid carcinoma cells (HeLa) as well as non-transformed (non-tumor) green monkey kidney cells (BGM) were selected as objects of study. The cells were obtained from Republican Scientific and Practical Center for Epidemiology and Microbiology of the Ministry of Health of the Republic of Belarus (Minsk) in the form of a suspension in the Dulbecco’s Modified Eagle’s Medium (DMEM) with 5% fetal serum. Cell monolayers were grown in disposable Petri dishes with a diameter of 35 mm on the same nutrient medium at 37 °C and 5% CO₂ in an incubator. An amount of 3 ml of growth medium with cells was added to each dish. The seed cell concentration was 130,000 ml⁻¹. After a period of 48 h following seeding, cell monolayers were exposed to blue light.

2.2 Effect of Optical Radiation on Cell Cultures

Cells in culture were exposed to blue light using semiconductor GaN lasers with radiation wavelengths of $\lambda = 405$ nm and $\lambda = 445$ nm. According to Ref. [82], the emission spectrum of semiconductor lasers of this type is formed by narrow lines with a total spectral width of ~1.5 nm. The lasers operated in continuous-wave mode. To obtain a more homogeneous light spot on the surface of cell monolayers, laser radiation was introduced into a monofilament quartz-polymer light guide with a light-conducting core diameter of 125 μ m. Petri dishes were irradiated from below. Light was defocused onto the entire surface of the bottom of Petri dish. The irradiance at the level of the surface of Petri dish bottom was varied in the range of $I = 5$ –100 mW/cm² and was controlled using a power meter PM100D with an S121C photodiode sensor (Thorlabs GmbH, Germany). Irradiation time was varied in the range of 1–10 min. After irradiation, the cells were placed for 24 h in a CO₂ incubator, which maintained the temperature at 37 °C and 5% CO₂ content. Similar manipulations (except for irradiation) were performed with control cell monolayers.

2.3 Influence of Light on Metabolic Activity of Cells

The biological effect of optical radiation was assessed photocolorimetrically using MTT test. This test is based on the ability of dehydrogenases in living metabolically active cells to convert the pale yellow water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into blue formazan crystals, which are insoluble in water [83]. Non-viable dead cells do not have this ability. MTT produced by Applichem (Germany) was used in the studies. The MTT solution was prepared according to the standard procedure: 5 mg of MTT was dissolved in 1 ml of H₂O (concentrated solution). After 24 h of cell incubation after irradiation, the growth

medium was spilled out and a new one without serum, containing 50 μl of concentrated MTT per 1 ml of medium, was added. The cells were incubated with new medium for 3 h at 37 °C and 5% CO_2 . After 3 h of incubation, the supernatant was removed and the cell monolayer was left in Petri dishes overnight to dry. Then, 1 ml of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to each Petri dish and incubated for 30 min at room temperature. Analysis of the metabolic activity of cells was carried out by monitoring the absorbance of extract at a wavelength $\lambda = 570 \text{ nm}$. A quantitative measure of the metabolic activity of cells after exposure to light in comparison with control samples was the value $\gamma = (A_{ir}/A_c) \cdot 100\%$, where A_{ir} and A_c are the absorbance of formazan solution from experimental (irradiated) and control Petri dishes, respectively.

2.4 Chemiluminescence Assay for Monitoring the Formation of Reactive Oxygen Species upon Exposure to Optical Radiation

To determine the contribution of reactive oxygen species to the photobiological effects initiated by exposure of cells to blue light, chemiluminescence assay was used. As is known [84, 85], the chemiluminescence is quite sensitive, since it allows simultaneous (total) detection of various reactive oxygen species (including superoxide anion radical $\text{O}_2^{\bullet-}$, hydrogen peroxide H_2O_2 and hydroxyl radical $\text{OH}^{\bullet}$), localized both in the intracellular and in extracellular space. Chemiluminescence parameters were measured using a Lum 5773 chemiluminometer (DISoft, Russia), operating in photon counting mode with a spectral sensitivity range of 300–650 nm. Registration and processing of chemiluminescence signals was carried out using specialized software “Power Graph 3.3 Professional”.

To study the effect of radiation on cell chemiluminescence, a cell suspension with a concentration of $2 \cdot 10^6 \text{ ml}^{-1}$ was used. An amount of 220 μl of suspension was irradiated in glass cylindrical cuvettes with an internal diameter of 10 mm and a flat bottom. The suspension was irradiated through the bottom of the cuvette by lasers with a wavelength of 405 or 445 nm. The irradiation time was $t = 3 \text{ min}$, irradiance at the surface level of the cuvette bottom was $I = 50 \text{ mW/cm}^2$. After cessation of irradiation, the suspension was immediately transferred to a chemiluminometer cuvette. The time from cessation of irradiation to the start of recording the chemiluminescence signal was strictly controlled and was 24 s. Similar manipulations (except for irradiation) were carried out with control samples. Measurements of chemiluminescence of control (non-irradiated) and experimental samples of cell suspensions was alternated in a random order. The luminometer measured chemiluminescence (in relative units) at 22 °C for 5 min after the start of recording.

To determine the type of reactive oxygen species, involved in photochemical processes, initiated by exposure to blue light, 10 μl of one of the specific

quenchers (scavengers) of reactive oxygen species was added to the cell suspension 10 min before irradiation: sodium azide – a quencher of singlet oxygen, sodium pyruvate – a hydrogen peroxide scavenger or D-mannitol – a hydroxyl radical scavenger. The concentration of stock solutions of the indicated quenchers (scavengers) was prepared in such a way that after adding 10 μl of one of them to 220 μl of a cell suspension, the final concentration were as follows: 10 mM for sodium azide and sodium pyruvate, 40 mM for D-mannitol. To account for dilution, 10 μl of saline solution was added to samples of cell suspensions that did not contain ROS quenchers (scavengers).

2.5 Registration of Fluorescence of Endogenous Porphyrins in Cell Extracts

As is known, the fluorescence of endogenous porphyrins in a suspension of somatic cells is very difficult to detect due to the predominant luminescence of flavins and flavoproteins, which is due to their higher concentration (exceeding the concentration of porphyrins by about 2–3 orders of magnitude) and a higher (5–10 times) fluorescence quantum yield [29, 77]. More reliably, the fluorescence of free-base porphyrins and their zinc complexes is recorded after the extraction of tetrapyrroles from cells using 3 M hydrochloric acid. This is due to the good solubility of porphyrins in hydrochloric acid and the multidirectional effect of hydrochloric acid on the fluorescence intensity of flavins (the fluorescence of flavin mononucleotide after treatment with 3 M HCl is quenched by more than 60 times) and porphyrins (the fluorescence of protoporphyrin IX increases by more than 20 times compared to an aqueous solution) [29, 77]. A detailed description of methods for extracting porphyrin fluorophores from the studied cell cultures is presented in Refs. [29, 77]. We only note that, when performing comparative studies of porphyrin fluorescence of acidic extracts of HeLa and BGM cells, their initial concentration in saline solution was controlled either using a hemocytometer or a flow cytometer and adjusted by appropriate dilution with saline solution to the same value of $5 \cdot 10^5 \text{ cells/ml}$ for each type cells. Moreover, all subsequent manipulations for the extraction of porphyrins from HeLa and BGM cells according to the method [29, 77] were of the same type.

Absorption spectra were measured on a Cary-500 ScanUV-Vis-NIR spectrophotometer (Varian, USA, Australia), fluorescence emission and fluorescence excitation spectra were measured on a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon, Inc., France), using standard quartz cuvettes of $10 \times 10 \times 40 \text{ mm}$, as well as special cuvettes with an optical path length of $4 \times 4 \text{ mm}$. The width of monochromator slits was 5 nm.

2.6 Statistical Analysis

Statistical analysis was performed using Statistical Analysis System software (SAS Enterprise Guide, version 7.1, SAS Inst. Inc.). Data were tested for normal

distribution (Shapiro-Wilk test) followed by pairwise comparisons using Student's *t*-test. All data are presented as mean $\pm$ standard error of the mean. A *p*-value < 0.05 was chosen to indicate statistical significance. The results presented were obtained from at least 3 independent experiments.

3 Results

3.1 Effect of Blue Light on Metabolic Activity of Cancer and Normal Cells

Studies have shown that the effect of laser radiation of blue spectral region with $\lambda = 405$ nm or 445 nm and irradiance of $I = 25$ mW/cm² in the energy range doses of $D = 1\text{--}15$ J/cm² on cells of various types (BGM, HeLa) is inhibition of their metabolic activity, controlled 24 h after cessation of irradiation. The results obtained are presented in Fig. 1(a, b).

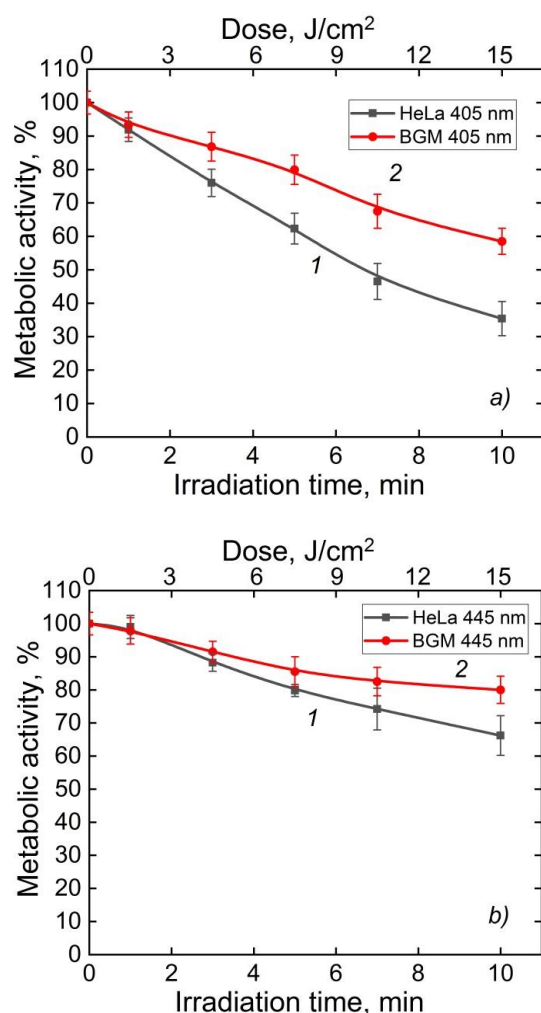


Fig. 1 Dose-dependent metabolic activity of (1) HeLa and (2) BGM cells as a percentage of the control 24 h after exposure to laser radiation with wavelength of $\lambda = 405$ nm (a), 445 nm (b) at irradiance of $I = 25$ mW/cm². The lower axis shows the time of irradiation of cell monolayer in minutes, the upper axis shows the dose of radiation in J/cm².

It follows from the Fig. 1 that as energy dose of light exposure increases, an increase in the degree of inhibition of the metabolic activity of both HeLa and BGM cells is observed. However, at the same energy dose, the inhibitory effect for radiation with wavelength of $\lambda = 405$ nm is significantly higher in relation to HeLa cells (Fig. 1(a), curve 1) than in relation to BGM cells (Fig. 1(a), curve 2). A higher sensitivity of cancer cells (Fig. 1(b), curve 1) compared to normal (Fig. 1(b), curve 2) is also registered when exposed to radiation with wavelength of $\lambda = 445$ nm. It is also characteristic that after exposure to radiation $\lambda = 405$ nm, the rate of inhibition of metabolic activity of cells is higher than after exposure to radiation $\lambda = 445$ nm. This pattern appears for both cancer and normal cells.

3.2 Effect of Blue Light on Efficacy of Generation of Reactive Oxygen Species in Cancer and Normal Cells

As we have shown previously in experiments with various types of cells [29, 74, 75], important information about photochemical processes occurring in cells can be obtained using chemiluminescence assay, which makes it possible to collectively detect various reactive oxygen species (including superoxide anion radical $O_2^{\bullet-}$, hydrogen peroxide H_2O_2 and hydroxyl radical $OH^{\bullet}$), localized both in intracellular and extracellular spaces.

Chemiluminescence intensity kinetic curves for suspension of HeLa (A, B) and BGM (C, D) cells in saline when it is recorded for 5 min in the case of using control (non-irradiated) cell samples (A and C), as well as samples previously exposed to radiation (B, D) $\lambda = 405$ nm, irradiance $I = 50$ mW/cm², for $t = 180$ s (energy dose – $D = 9.0$ J/cm²) are presented in Fig. 2. The concentration of HeLa and BGM cells was $2 \cdot 10^6$ ml⁻¹.

Analysis of the data presented in Fig. 2 shows the presence of significant differences in the course of kinetic curves of chemiluminescence intensity of irradiated and non-irradiated cell suspensions. These differences are manifested primarily in the fact that the kinetic curves of changes in chemiluminescence intensity for irradiated cell suspensions (B, D) are descending curves, while the average chemiluminescence intensity for control (intact) cell suspensions (A and C) remains unchanged throughout the entire signal recording period. In addition, the amplitude of signal fluctuations relative to the average curve approximating the kinetics of signal changes is significantly higher for irradiated cells.

However, the main conclusion that follows from the kinetic curves of chemiluminescence intensity of irradiated samples of cell suspensions is that, at the same concentration in the suspension of HeLa (B) and BGM cells (D), the integrated signal intensity (chemiluminescence light sum) is reliably higher in case of preliminary exposure of cancer cells to light.

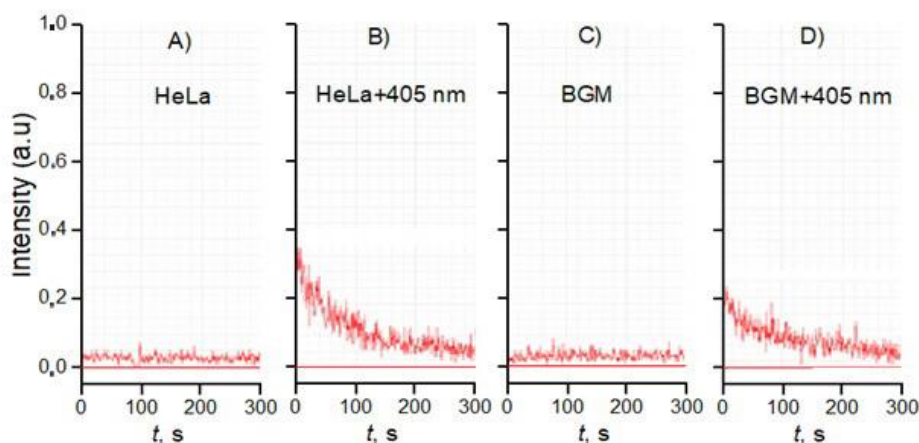


Fig. 2 Chemiluminescence intensity kinetic curves for suspension of (A, B) HeLa and (C, D) BGM cells with concentration of $2 \cdot 10^6 \text{ ml}^{-1}$ in saline solution: (A) and (C) control cell suspensions not exposed to optical radiation; (B) and (D) cell suspensions after exposure to laser radiation with wavelength of $\lambda = 405 \text{ nm}$, irradiance $I = 50 \text{ mW/cm}^2$, for $t = 180 \text{ s}$ (dose $D = 9.0 \text{ J/cm}^2$).

Since the presence of chemiluminescence signal as a result of exposure to light is explained by the occurrence of photochemical reactions involving various types of ROS, the results obtained indicate higher levels of light-induced ROS formation in HeLa cells compared to normal BGM cells.

3.3 Chemiluminescence Assay to Study the Mechanism of Light-Induced Damage of HeLa Cells

Using chemiluminescence assay, it was possible to show that the main type of reactive oxygen species that initiates damage to cancer cells when exposed to blue light is singlet oxygen. This conclusion follows from the results of studying the effect of specific ROS quenchers (scavengers) (sodium azide – a quencher of singlet oxygen, sodium pyruvate – a scavenger of hydrogen peroxide, D-mannitol – a scavenger of hydroxyl radicals), which were added to the cell suspension 10 min before its irradiation, on the chemiluminescence light sum of cell suspension. It is important to note that all the quenchers (scavengers) of reactive oxygen species used in this work do not absorb at wavelengths of the acting radiation $\lambda = 405$ and 445 nm . Therefore, the change in the magnitude of the light-induced effect in cells cannot be associated with screening of the acting radiation by quenchers (scavengers). The results obtained are presented in Fig. 3, in which the integral, the area (S) under the chemiluminescence curve from the beginning of recording ($t = 0 \text{ s}$) to $t = 300 \text{ s}$, is taken as a parameter characterizing the integral intensity of chemiluminescence (see Fig. 2).

As follows from the Fig. 3, for all non-irradiated samples of cell suspensions, not containing (Contr) and containing (NaN_3 , SP, Man) quenchers of ROS, the integral intensity of chemiluminescence is recorded at approximately the same low level ($S_{\text{Contr}} \rightarrow S_{\text{NaN}_3} \rightarrow S_{\text{SP}} \rightarrow S_{\text{Man}}$). Consequently, the addition of ROS quenchers

(scavengers) has no effect on the spontaneous (not initiated by light) chemiluminescence of HeLa cell suspension. However, after 180 s exposure of cell suspension, not containing quenchers, to laser radiation with wavelength of $\lambda = 405 \text{ nm}$, $I = 50 \text{ mW/cm}^2$, $D = 9.0 \text{ J/cm}^2$ (Ir), the integral signal intensity increases significantly, reaching the value $S_{\text{Ir}} = 1.0$. The addition of singlet oxygen quencher, sodium azide, to the cell suspension before irradiation followed by exposure to blue light of the same parameters ($\text{NaN}_3 + \text{Ir}$) leads to a significant increase in the signal compared to the non-irradiated control, containing the specified quencher (NaN_3).

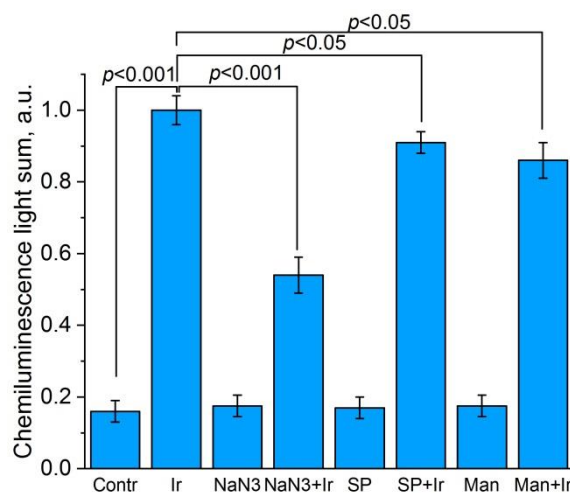


Fig. 3 Integral chemiluminescence of HeLa cell suspension without irradiation and without external additives (Contr); without irradiation in the presence of 10 mM sodium azide (NaN_3), 10 mM sodium pyruvate (SP), 40 mM D-mannitol (Man); irradiated suspension in the absence of external additives (Ir), in the presence of 10 mM sodium azide ($\text{NaN}_3 + \text{Ir}$), 10 mM sodium pyruvate (SP + Ir) or 40 mM D-mannitol (Man + Ir). Parameters of optical radiation: $\lambda = 405 \text{ nm}$, $I = 50 \text{ mW/cm}^2$, duration $t = 180 \text{ s}$, $D = 9.0 \text{ J/cm}^2$.

However, compared to the irradiation without additives (Ir), the integral intensity of chemiluminescence of the cell suspension, irradiated in the presence of NaN_3 ($\text{NaN}_3 + \text{Ir}$) is lower by 46%, $S_{\text{NaN}_3 + \text{Ir}} = 0.54$. A decrease in the magnitude of the photobiological effect in the presence of singlet oxygen quencher indicates the participation of this form of oxygen in photochemical processes in HeLa cells.

Unlike sodium azide, the addition of hydrogen peroxide scavenger, sodium pyruvate, followed by exposure to blue light has a significantly less effect on the integral intensity of the chemiluminescent signal ($S_{\text{SP}+\text{Ir}} = 0.91$) compared to the irradiation in the absence of quenchers ($S_{\text{Ir}} = 1.0$). Moreover, despite the slight differences in the indicated variants for irradiation of cell suspension, the participation of hydrogen peroxide in the photochemical processes initiated by blue light in cells is reliably recorded ($p < 0.05$).

As for the effect of hydroxyl radical scavenger – D-mannitol, as it follows from Fig. 3 that its addition to the cell suspension followed by exposure to light ($\text{Man} + \text{Ir}$) also leads to the registration of chemiluminescent signal that practically corresponds to the variant of cell irradiation without additives (Ir): $S_{\text{Man}+\text{Ir}} = 0.86$. Therefore, it can be assumed that when monitoring the participation of ROS in photodamage to HeLa cells immediately after the cessation of light exposure, hydroxyl radicals also play a certain role in the photochemical processes under study.

Thus, we can conclude that chemiluminescence initiated by exposure of cell suspension to blue light is mainly caused by generation of singlet oxygen in cells due to the excitation of endogenous photosensitizers and, to a lesser extent, due to processes involving hydrogen peroxide and hydroxyl radicals.

3.4 Determination of Type of Molecules Responsible for Formation of Chemiluminescence Signal upon Exposure of Cell Suspension to Laser Radiation with Wavelengths of $\lambda = 405 \text{ nm}$ and $\lambda = 445 \text{ nm}$

The next stage of our research was aimed at elucidating the type of molecules responsible for formation of chemiluminescence signal induced by exposure of cell suspension to blue light with wavelengths of $\lambda = 405 \text{ nm}$ and $\lambda = 445 \text{ nm}$. As already mentioned, there is reason to believe [29, 77] that this signal may be due to the participation of ROS in photochemical reactions in cells formed as a result of the excitation of free-base porphyrins (protoporphyrin IX, coproporphyrin III, uroporphyrin III) and their zinc complexes by blue light, as well as flavin molecules (riboflavin, RF, flavin mononucleotide, FMN, flavin adenine dinucleotide, FAD). As is known [86–88], these compounds in monomeric form are characterized by high efficacy of singlet oxygen generation and are also capable (albeit with much less efficacy) of generating other types of ROS [89]. The absorption spectra of one of the flavins

(FMN), protoporphyrin IX as well as Zn-protoporphyrin IX in monomeric form in DMSO are shown in Fig. 4. Note that the absorption spectra of RF, FMN and FAD are practically the same. For this reason, the presented data allow us to draw a conclusion about relationship between maxima in absorption spectra of above sensitizers of flavin and porphyrin nature.

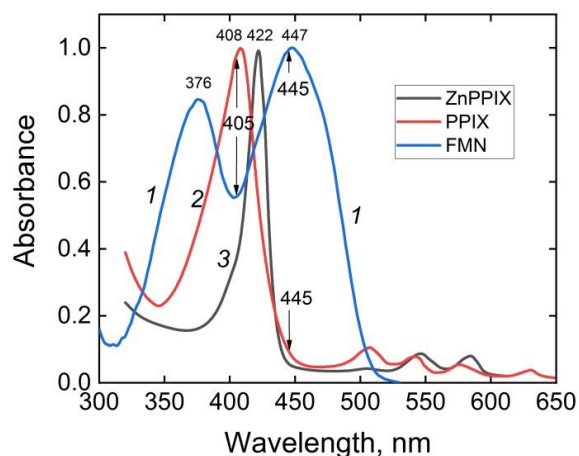


Fig. 4 Absorption spectra of endogenous photosensitizers: FMN in (1) buffer, (2) protoporphyrin IX and (3) Zn-protoporphyrin IX in DMSO. Arrows are wavelengths of radiation acting on cells

From Fig. 4 it follows that the absorption spectra of flavins in aqueous environment are characterized by two maxima: short-wavelength maximum in the region of 376 nm and long-wavelength maximum in the region of 447 nm, as well as a local minimum in the region of 405 nm. Protoporphyrin IX is characterized by the presence of an intense absorption band (Soret band, $S_2 \leftarrow S_0$ – transition) in the region of $\lambda_{\text{max}} = 408 \text{ nm}$ ($B(0,0)$) and four significantly weaker Q-bands ($S_1 \leftarrow S_0$ – transition) in the green, yellow and red spectral regions with maxima at $\lambda_{\text{max}} = 507 \text{ nm}$ ($Q_y(1,0)$), $\lambda_{\text{max}} = 543 \text{ nm}$ ($Q_y(0,0)$), $\lambda_{\text{max}} = 575 \text{ nm}$ ($Q_x(1,0)$), $\lambda_{\text{max}} = 630 \text{ nm}$ ($Q_x(0,0)$). In other words, laser radiation with wavelength of $\lambda = 405 \text{ nm}$ corresponds to the maximum of absorption band of protoporphyrin IX and to the region of the local minimum of absorption spectrum of flavins. At the same time, laser radiation with wavelength of $\lambda = 445 \text{ nm}$ corresponds to the maximum absorption of flavins and to the region of local minimum of the absorption spectrum of protoporphyrin IX. Moreover, the absorption coefficient of porphyrin in the region of 445 nm is approximately 20 times lower than its value at 405 nm.

The absorption spectrum of Zn-protoporphyrin IX in DMSO is characterized by the presence of intense Soret band ($S_2 \leftarrow S_0$ – transition) with maximum at $\lambda_{\text{max}} = 422 \text{ nm}$ ($B(0,0)$) and two weaker Q-bands ($S_1 \leftarrow S_0$ – transition) with maxima at $\lambda_{\text{max}} = 547 \text{ nm}$ ($Q(1,0)$), $\lambda_{\text{max}} = 584 \text{ nm}$ ($Q(0,0)$). From Fig. 4 it follows that Q-absorption bands of Zn-protoporphyrin IX are located outside the absorption spectrum of flavins, and the Soret band falls on the short-wavelength slope of their long-wavelength absorption band.

Naturally, the contribution of one or another endogenous acceptor to photosensitized damage of cellular structures depends on a number of factors: (a) the ratio of absorption coefficients of acceptors at the wavelength of the influencing radiation; (b) ratio of acceptor concentrations; (c) their photosensitizing properties (for example, the efficacy of ROS generation); (d) the presence near the sensitizer of biologically important molecules capable of interacting with ROS or triplet-excited sensitizer (in photochemical processes by a radical mechanism - type I mechanism); (e) the presence in the immediate vicinity of the sensitizer of ROS quenchers (scavengers) or quenchers of the triplet-excited state of the photosensitizer.

From the data presented in Fig. 4, it is clear that in the blue spectral region, in the case of a noticeable role of porphyrins in the effects of sensitization, one should expect that their contribution will be most pronounced in the region of the local minimum of absorption band of flavins ($\lambda_{\min} = 405$ nm), since in this region one of the porphyrin photosensitizers (protoporphyrin IX) is characterized by an intense band with a maximum at $\lambda_{\max} = 408$ nm. In this regard, it was of interest to compare the efficacy of ROS formation when exposed to laser radiation with wavelength of $\lambda = 405$ nm, corresponding to the long-wave absorption maximum of protoporphyrin IX, and radiation with a wavelength of $\lambda = 445$ nm, corresponding to the maximum in the absorption spectrum of flavins.

Results of comparative studies of chemiluminescence light sum (in relative units) induced by exposure of suspension of HeLa cells to laser radiation with wavelength of $\lambda = 405$ nm and $\lambda = 445$ nm for $t = 180$ s, irradiance $I = 50$ mW/cm² ($D = 9$ J/cm² energy dose) are presented in Fig. 5.

It follows from the Fig. 5 that the value of the light sum of chemiluminescence induced by exposure to radiation with $\lambda = 405$ nm significantly exceeds the corresponding indicator when exposed to radiation with $\lambda = 445$ nm. This confirms our assumption about the ability of endogenous porphyrins to contribute to photochemical processes in cells when exposed to blue light.

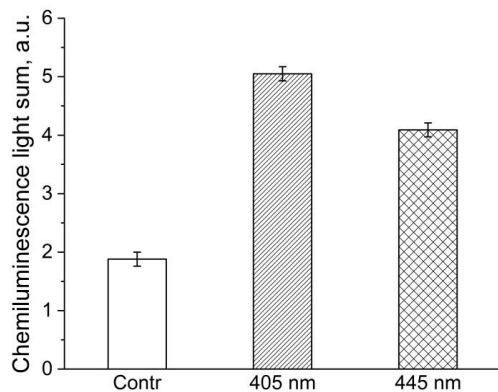


Fig. 5 Chemiluminescence light sum (a.u.) upon exposure of HeLa cell suspension to laser radiation $\lambda = 405$ nm and $\lambda = 445$ nm, irradiance $I = 50$ mW/cm² (dose $D = 9$ J/cm²) during $t = 180$ s.

Thus, the results obtained indicate that significant contribution to the processes affecting the metabolic activity of cells when exposed to laser radiation with $\lambda = 405$ nm (Fig. 1) can be made by endogenous porphyrins due to the sensitized formation of ROS, and, above all, singlet oxygen (Fig. 3). At the same time, the levels of ROS formation, controlled by chemiluminescence, are higher when HeLa cells are exposed to blue light compared to non-transformed BGM cells (Fig. 2), which are also characterized by lower photosensitivity to the action of this radiation compared to HeLa cells (Fig. 1).

3.5 Assessment of Relative Concentration of Endogenous Porphyrins in Cancer and Normal Cells Using Fluorescence Spectroscopy

To clarify the reasons for the increased photosensitivity of cancer cells to blue light, we performed comparative assessments of the relative concentrations of endogenous porphyrins in cancer and normal cells using fluorescent analysis. As already noted, due to the low concentration of endogenous porphyrins in cells (at the level of ~ 1 nM), the low quantum yield of their fluorescence ($\phi_f = 0.01$ – 0.08 [77]), high concentration of flavins in cells (0.3 – 1 μ M) [90, 91], higher quantum yield of their fluorescence ($\phi_f = 0.08$ – 0.26 [77]), as well as due to the overlap of absorption spectra of porphyrin and flavin photosensitizers, detection of porphyrin fluorescence in living cells is associated with significant difficulties. Fluorescence of endogenous porphyrins is more reliably recorded in acidic cell extracts [29, 77]. As was shown earlier [77], treatment of protoporphyrin IX with 3 M hydrochloric acid leads to an increase in the quantum yield of its fluorescence compared to aqueous solutions by approximately 20 times, while acid treatment of FMN is accompanied by a decrease in the quantum yield of its fluorescence by approximately 60 times. Naturally, such a multidirectional effect of HCl on the fluorescent characteristics of porphyrins and flavins improves the possibility of recording the luminescence of endogenous porphyrins in acidic cell extracts with an appropriate choice of the fluorescence excitation wavelength. The reasons for the multidirectional influence of hydrochloric acid on the spectral and fluorescent characteristics of flavins and free-base porphyrins are analyzed in detail in Ref. [77].

As an example, in Fig. 6, fluorescence emission spectra (curves 1 and 2) at excitation wavelength of $\lambda_{\text{ex}} = 405$ nm and fluorescence excitation spectra (curves 4 and 5) at registration wavelength of $\lambda_{\text{em}} = 656$ nm of acidic extracts of HeLa (curves 1 and 4) and BGM cells (curves 2 and 5) are shown. The fluorescence emission spectra (curve 3) and fluorescence excitation spectra (curve 6) of the extractant (3 M hydrochloric acid) are also shown.

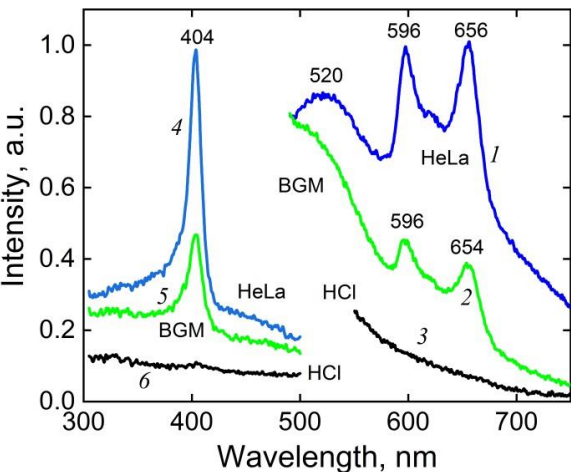


Fig. 6 Fluorescence emission (1–3) and fluorescence excitation (4–6) spectra of HeLa (1, 4), BGM (2, 5) cell extracts and extractant – 3 M hydrochloric acid (3, 6) at excitation wavelength of $\lambda_{ex} = 405$ nm (1–3) and registration wavelength of $\lambda_{em} = 656$ nm (4–6). Cell concentration for extraction was $5 \cdot 10^5$ ml⁻¹.

From the data presented it is clear that when fluorescence is excited by radiation with $\lambda_{ex} = 405$ nm, the emission spectrum of acidic cell extracts is characterized by a band with a pronounced maximum in the region of $\lambda_{max} = 520$ nm, caused by the fluorescence of flavin compounds present in acidic extracts and, above all, riboflavin [77]. On the long-wavelength slope of the flavin fluorescence band, a double-humped band with maxima at $\lambda_{max} = 596$ and 656 nm, characteristic for fluorescence of porphyrin compounds, is clearly detected. As was shown earlier [77], for extracts of HeLa cells in 3M HCl, the indicated fluorescence is closest to the fluorescence of chemically pure protoporphyrin IX in the indicated solvent.

In fluorescence excitation spectrum of cell extracts when using 3M HCl as an extractant and registration wavelength $\lambda_{em} = 656$ nm, a narrow band with a maximum at $\lambda_{max} = 404$ nm, characteristic of chemically pure protoporphyrin IX in hydrochloric acid, is clearly recorded [77].

As expected, there are no extreme points when recording the fluorescence emission spectra (curve 3) and fluorescence excitation spectra (curve 6) of a chemically pure extractant – 3M HCl (Fig. 6).

A comparison of fluorescence emission and fluorescence excitation spectra of HeLa (curves 1 and 4) and BGM (curves 2 and 5) cell extracts shows that they are qualitatively very similar. However, the intensity of the porphyrin fluorescence signal for HeLa cells is approximately 2.5 times higher than for normal BGM cells. The same quantitative pattern can be traced when analyzing the fluorescence excitation spectra of these types of cells. Since the fluorescence intensity is directly proportional to the concentration of fluorescent molecules, it can be concluded that the concentration of endogenous porphyrins in HeLa cells is approximately 2.5 times higher than in normal, non-transformed BGM cells. Taking into account the fundamental nature of the conclusion about the higher level of endogenous porphyrin concentrations in cancer cells, comparative studies of the intensity of porphyrin fluorescence for cancer and non-cancerous cells treated with hydrochloric acid were carried out in 5 pairs of independent experiments, which confirmed the above. Moreover, this conclusion was also confirmed by extracting endogenous porphyrins from cells using acetone, which, according to Ref. [77], allows identifying porphyrins in cancer cells.

4 Discussion

As already noted, in the overwhelming number of experimental studies that compared the effect of blue light from laser, LED or lamp sources on cancer and normal cells, it was found that at equal spectral and energy parameters of specified physical factor, a higher degree of photoinhibition of metabolic activity of cancer cells is noted [4, 8, 23, 31, 36, 39, 46, 48, 49, 57, 59, 60], as well as higher levels of light-induced ROS production [23, 28, 60].

The results of comparative studies of the effect of blue light on cancer and normal cells available in the literature are summarized in Table 1.

Table 1 Influence of blue light on rate of cell proliferation, ROS level and apoptosis of cancer and normal cells.

Type of cancer cells	Type of normal cells	Source of light, wave-length	Irradiance, mW/cm ² , Fluence, J/cm ²	Rate of cell proliferation as percentage to control, %			Light-induced ROS formation (a.u.)		Apoptosis, %		Ref.
				Cancer cells			Cancer cells	Normal cells	Cancer cells	Normal cells	
HT29 and HCT116 human colon cancer cells	CSC-2FO human fibroblast cells	LED, 465 nm	30 mW, 10 min/day during 5 days	HT29	HCT116	CSC-2FO					[4]
				24.8	37.1	~100					
HCT116 and HT29 colorectal cancer cells	MRC-5 human embryonic lung fibroblast	LED, 465 nm	3×10^4 lux, 2 h	HT29	HCT116	MRC-5					[8]
				~50	~50	~100					
OSC2 gingival (oral) squamous carcinoma cells	NHEK normal human pooled primary epidermal keratinocytes	QTH [#] , 380–500 nm	5 J/cm ² 15 J/cm ² 60 J/cm ²	OSC2	–	NHEK	OSC2	NHEK	OSC2	NHEK	[23]
				90	–	130	–	–	detected	Not detected	
				–	–	–	125	100	–	–	
				20	–	120	–	–	–	–	

Table 1 Cont.

OSC2 gingival (oral) squamous carcinoma cells	NHEK normal human epidermal keratinocytes	QTH [#] , 380–500 nm	550 mW/cm ² 15 J/cm ² 30 J/cm ²		OSC2	28 12 14	3 3	[28]
HeLa human cervical cancer cells	hDF human dermal fibroblasts	LED, 450 nm	25700 cd/m ² , 30 min after 24 h	HeLa 62	hDF 110			[31]
SYO-1, HS-SY-II, Saka-SS, Yamato-SS synovial sarcoma cells	HEK293 human embryonic kidney cell line	LED, 465 nm	48 h 0.6 mW/cm ² 48 h 0.6 mW/cm ²	SYO-1 45 Saka-SS 33	HS-SY-II 70 Yamato-SS 58	HEK293 80 – –		[36]
U937 tumor cells with diffuse histiocytic lymphoma	PBMC peripheral blood mononuclear cells	LED, 456 nm	0.25 mW/cm ² 2 h	U937 38	–	PBMC 90		[39]
PaCa-2, PANC-1, BxPC-3 human pancreatic cancer cells	H6c7 human pancreatic duct epithelial cell line; HUVEC human umbilical vein endothelial cells	LED, 460 nm	5 h/day for 5 days 5 mW/cm ² 10 mW/cm ² 5 h/day for 5 days 5 mW/cm ² 10 mW/cm ²	PaCa-2 52 30 – – –	PANC-1 41 42 BxPC-3 54 55	H6c7 83 78 HUVEC – 80		[49]
SRSY5Y human neuroblastoma cancer	HDF human dermal fibroblasts	LED, 460–470 nm	10,540 lux 6 h irr. + 12 h dark+6 h irr. after 24 h after 48 h after 72 h	– 83 91 91	SRSY5Y 83 96 97	HDF 83 96 97	SRSY5Y 2.70 26.60	[57]
PC-3 prostate cancer cells, A2780 ovarian carcinoma cells, G361 malignant melanoma cells	PNT1A prostatic epithelial tissue cells	plasma light source, 485 nm, bandwidth 25 nm	208 mJ/cm ² 208 mW/cm ² ·1s	PC-3 $k^{\#} = -0.53$ G361 $k^{\#} = 0^{\#}$	A2780 $k^{\#} = -2.12$	PNT1A $k^{\#} = 3.38$		[59]
A549 human lung carcinoma cells	MRC-5 human lung fibroblast	diode-pumped solid state laser, 473 nm	750 mW/cm ² 5 min			A549 44	MRC-5 15	[60]
erythroblastic leukemic cells (EBL)	peripheral blood lymphocytes (PBL)	LED, 470 nm	5.7 mW/cm ² , 3 h Day 0 Day 4 Day 7	EBL 100 19.0 18.3	PBL 100 67.5 79.3			[46]
MCF-7 human epithelial breast carcinoma, OSC-2 human oral squamous cell carcinoma keratinocytes	NHEK normal human foreskin keratinocytes, WI-38 human lung fibroblasts, Balb/c 3T3 mouse lung fibroblasts, HGF human gingival fibroblasts	Ar-laser 400–500 nm	202 mW/cm ² 60 J/cm ²	MCF-7 86	OSC-2 92	NHEK 130 Balb/c 3T3 5 WI-38 145 HGF 150		[33]

Table 1 Cont.

			MCF-7	OSC-2	NHEK	
			79	14	128	
					Balb/c 3T3	
					0	
					WI-38	
					87	
					HGF	
					83	
			MCF-7	OSC-2	NHEK	
			18	5	104	
					Balb/c 3T3	
					0	
					WI-38	
					122	
					HGF	
					82	
T24 and EJ human bladder cancer cells	SV-HUC-1 human uroepithelial cells	diode laser, 450 nm	100 mW/cm ² 20–240 s 2, 4, 8, 12, 16, 20, 24 J/cm ²	T24	EJ	SV-HUC-1
			IC ₅₀ [#] after 24 h, J/cm ²	9.8	14.6	20.0
			IC ₅₀ [#] after 48 h, J/cm ²	8.0	8.9	17.9

[48]

[48]

QTH[#] – dental quartz-tungsten-halogen lamp; 94% power of radiation in the 400–500 nm range [58];
PAC[#] – plasma arc curing lamp, 81 % power of radiation in the 400–500 nm range [58];
 $k^{\#}$ – the slope of the approximating line, reflecting the change in the number of cells during 24 h of the experiment (increase in value per hour);
IC₅₀[#] – half maximal inhibitory energy dose (an energy dose that reduces the proliferative activity of cells by half).

From the analysis of the literature data, it follows that most often comparison of the effect of blue light on cancer and normal cells was carried out by monitoring the light-induced change in metabolic activity (viability) of the cells [4, 8, 23, 31, 36, 48], their proliferation [4, 31, 39, 46, 49, 57, 59] and migration [36, 48, 57, 59, 60], as well as changes in cell cycle parameters [4, 49, 57].
Most of the studies presented in Table 1, and confirming the higher sensitivity of cancer cells to blue light, were performed using LED sources [4, 8, 29, 31, 36, 39, 46, 49, 57]. However, this conclusion is also true when cells are exposed to laser radiation, which follows from the data presented in the present work and in Refs. [33, 48, 60], as well as a quartz-tungsten-halogen lamp [23, 28, 33] and a plasma arc curing lamp [59]. Thus, the more pronounced effect of blue light on cancer cells compared to non-cancerous cells is not associated with the laser specificity of the characteristics of optical radiation. Moreover, as shown in Ref. [29], there are no fundamental differences in the effect of laser radiation and radiation from LED sources emitting in the blue spectral region on cells. For example, according to Ref. [29], when exposed to laser radiation with $\lambda = 405$

nm and an LED source with $\lambda_{max} = 405$ nm, for which full width at half maximum FWHM = 17 nm, the changes in the metabolic activity of cells are equivalent. The same conclusion follows when comparing the photobiological effects initiated by laser radiation with $\lambda = 445$ nm and radiation from an LED source with $\lambda_{max} = 445$ nm, FWHM = 18 nm [29].
Some of the above studies note that while there is a significant inhibitory effect of blue light with wavelengths of 450, 456, 460, 465, 470, 473, 485, 380–500, 450–500 nm on the proliferation and migration of cancer cells, its effect on normal cells are practically absent [4, 23, 31, 46, 49, 59, 60]. Other studies [8, 33, 39, 48, 57] show the presence of photobiological effect when exposed to blue light with wavelengths of 450, 465, 460–470, 400–500, 450–500 nm on both cancer and normal, non-transformed cells. Moreover, according to Refs. [8, 33, 39, 48, 57], the inactivating effect of blue light is more pronounced against cancer cells. For example, according to Ref. [39], irradiation of human leukemic monocytic lymphoma cells U937 with LED source peaking at $\lambda_{max} = 56$ nm for $t = 2$ h at irradiance of $I = 0.25$ mW/cm² (energy dose was $D = 1.8$ J/cm²) leads

to a decrease in proliferation by 75%, while the same effect on normal non-transformed peripheral blood mononuclear cells is accompanied by a decrease in the corresponding proliferation rates by only 10%.

In our opinion, the absence of effect of blue light on normal cells in studies [4, 23, 31, 46, 49, 59, 60] is due to the insufficiently high energy dose of light exposure used. This is indicated by numerous studies of effect of blue light of the above wavelengths on non-tumor cells, which leave no doubt about its ability to influence the functional characteristics of this type of cell [1, 92]. In addition, the authors [48] showed that in two bladder cancer cell lines upon exposure to laser radiation with wavelength of $\lambda = 450$ nm, a decrease in their viability begins to be recorded at an energy dose of $D > 4$ J/cm², while for non-cancer uroepithelial cell viability begins to decrease at $D > 16$ J/cm². In other words, the photobiological effect, which was absent in relation to normal cells at low energy doses, begins to appear at higher doses.

At the same time, it should be noted that some reports [27] indicate the absence of significant differences in the effect of blue light ($\lambda = 465$ nm, energy dose was $D = 123$ J/cm² per day, total dose for three days of cell irradiation was $D = 369$ J/cm²) on cancer and normal cells, and according to certain indicators (decrease in viability, percentage of cells in the apoptosis stage), the photobiological effect on normal cells according to Ref. [27] is higher than the corresponding value for cancer cells. In our opinion, one of the reasons for the fundamental differences between these results and the data of other authors may be the excessively high dose on the cells, which is almost 50 times higher than the doses usually used in other literature sources. However, despite this work, the totality of data contained in Table 1 strongly suggests a higher sensitivity of cancer cells to blue light compared to normal, non-transformed cells.

In this regard, it is noteworthy that the higher sensitivity of cancer cells compared to normal cells has also been confirmed with cells obtained from the same organ [33, 48, 59]. Thus, in studies [33], performed using a lamp source emitting in the range of 400–500 nm, or an argon laser generating radiation in the range of 450–500 nm, it was shown that the decrease in survival (energy dose was $D = 60$ J/cm²) is strongly pronounced against cancer cells (carcinoma) of the oral cavity, while the inhibitory effect is barely registered against normal cells of the same location.

The same results were obtained by the authors [59] when monitoring, using a multimodal holographic microscope, the migration of cancer and normal prostate cells, previously exposed to lamp source (halogen lamp) with wavelength of $\lambda = 485 \pm 12.5$ nm at an energy dose of $D = 0.208$ mJ/cm².

As already noted, a higher sensitivity of bladder cancer cells compared to normal uroepithelial cells was established in Ref. [48] when exposed to semiconductor laser radiation with wavelength of $\lambda = 450$ nm in the energy dose range of $D = 4$ –24 J/cm².

The results of our research, presented in Fig. 1, are in full accordance with most of the above-cited works. From these data it follows that the rate of light-induced decrease in the metabolic activity of cancer cells is higher than that of normal cells both when exposed to radiation $\lambda = 405$ nm and $\lambda = 445$ nm. Moreover, the differences in the magnitude of the inhibitory effect of light between these types of cells increase as the energy dose increases. However, at initial stage of dose dependence, these differences are minimal, which is consistent with the results of Ref. [48].

And the second important conclusion, following from the results of Fig. 1, is that the rate of decrease in metabolic activity of both cancer and normal cells is significantly higher when exposed to radiation with a wavelength of $\lambda = 405$ nm compared with $\lambda = 445$ nm.

At the same time, our studies using chemiluminescence assay (Fig. 2 and Fig. 3) showed that reactive oxygen species, and, above all, singlet oxygen, play an important role in reducing the metabolic activity of cells when exposed to blue light. This is indicated by a significant decrease in the chemiluminescence signal when a specific singlet oxygen quencher, sodium azide, is added to the cell suspension before irradiation. In addition to singlet oxygen, hydrogen peroxide and hydroxyl radicals also play a certain role in photochemical processes in cells when exposed to blue light, since the addition of a specific hydrogen peroxide scavenger, sodium pyruvate, or hydroxyl radicals scavenger, D-mannitol, to the cell suspension before irradiation leads to reliable decrease in the chemiluminescence signal (Fig. 3).

The results obtained confirming the participation of ROS in photochemical processes in cells exposed to blue light are in good agreement with numerous literature reports [7, 8, 16, 19–21, 23, 28, 30, 35, 36, 38, 41, 60], in which the formation of ROS was detected using specific fluorescent probes. At the same time, our studies have shown (Fig. 2) that with the same spectral and energy characteristics of laser radiation ($\lambda = 405$ nm, $I = 50$ mW/cm², $t = 180$ s, $D = 9$ J/cm²) the light sum of chemiluminescent signal is significantly higher for cancer cells than for normal cells. This result is in accordance with the data of other authors [23, 28, 60]. Thus, studies [23] show that the level of ROS formation, assessed using a fluorescent probe, in epithelial carcinoma cells upon exposure to broadband blue light ($\lambda = 380$ –500 nm, $D = 60$ J/cm²) from lamp source at 30% higher than when irradiating normal epithelial keratinocytes (see Table 1).

Even more significant differences in level of light-induced ROS formation in the same types of cancer and normal cells, studied in Ref. [23], are reported by the authors [28] of the same research group when exposed to radiation $\lambda = 380$ –500 nm, but at energy doses $D = 15$ and 30 J/cm².

Thus, the results of our studies, obtained using chemiluminescence assay, indicating a higher level of light-induced ROS formation in cancer cells compared to normal cells, are fully consistent with literature data

based on recording the fluorescence of specific probes in cancer and normal cells.

In addition to studies confirming higher levels of light-induced ROS formation in cancer cells, we also performed comparative studies of ROS levels in HeLa cells exposed to laser radiation with wavelength of $\lambda = 405$ nm, located in the region of the maximum of the Soret band of protoporphyrin IX, and laser radiation with wavelength of $\lambda = 445$ nm, corresponding to the long-wave maximum of absorption spectrum of flavins (Fig. 4). In this case, radiation with wavelength of $\lambda = 405$ nm corresponds to the local minimum in absorption spectrum of flavins, and radiation with $\lambda = 445$ nm corresponds to the region of local minimum in absorption spectrum of porphyrins.

As follows from the results presented in Fig. 5, the level of ROS formation induced by exposure of suspension of HeLa cells to radiation with $\lambda = 405$ nm is significantly higher than when exposed to radiation with $\lambda = 445$ nm at equal energy parameters ($I = 50$ mW/cm², $t = 180$ s, $D = 9$ J/cm²). In our opinion, the result obtained is a strong argument indicating the contribution of porphyrins to the effects of sensitized ROS formation when cells are exposed to blue light. Since, in the case of the predominant role of flavins in the effects of sensitization, one would expect a higher level of ROS formation when cell suspension is exposed to radiation $\lambda = 445$ nm, corresponding to the long-wave maximum of absorption spectrum of flavins and the region of local minimum in absorption spectrum of porphyrins (Fig. 4). In addition, in the case of the predominant role of flavins in sensitized (due to endogenous compounds) reactions in cells, one would expect a higher rate of decrease in the metabolic activity of cells when exposed to radiation $\lambda = 445$ nm compared to radiation $\lambda = 405$ nm. However, as follows from Fig. 1, the rate of decrease in metabolic activity of both cancer and normal cells when exposed to radiation with $\lambda = 405$ nm significantly exceeds this indicator when cell suspension is exposed to radiation with $\lambda = 445$ nm.

It should be noted that the results of our studies, indicating a greater sensitivity of HeLa cells to radiation with $\lambda = 405$ nm compared to longer wavelength light with $\lambda = 445$ nm, are in good agreement with the results of the authors [93], who studied effect of low-intensity blue light on DNA synthesis in HeLa cells. These studies have shown that of the four wavelengths of a mercury-quartz lamp $\lambda_{max} = 313, 365, 404$ and 434 nm (selected from its emission spectrum using a monochromator) with an energy dose of $D = 1$ mJ/cm², the maximum stimulating effect on DNA synthesis in cells is observed at wavelength of 404 nm. In other words, studies [93] have shown that light with a wavelength corresponding to the absorption maximum of protoporphyrin IX and the local minimum in the absorption spectrum of flavins is characterized by the greatest biological activity. This confirms our hypothesis about the ability of endogenous porphyrins to act as one of the acceptors that, after absorbing light, can initiate a cascade of photochemical reactions affecting the course of metabolic processes in

the cell. Note that the same results were obtained when studying the spectral dependence of the inactivation effect on normal non-transformed somatic cells [94, 95]. Thus, according to data [94], which studied the effect of LED radiation with $\lambda_{max} = 410, 420, 453$ and 480 nm on fibroblasts, the greatest effect at energy doses $D = 15, 30, 60, 90$ J/cm² on cell viability and proliferation, as well as the generation of reactive oxygen species was caused by radiation with $\lambda_{max} = 410$ nm. Cytotoxicity was not observed when exposed to light with $\lambda_{max} = 453$ and 480 nm. And, in studies [95] of the cytotoxicity of blue light with $\lambda_{max} = 412, 419, 426$ and 453 nm at energy doses $D = 33, 66, 100$ J/cm², as well as its effect on the proliferation of human keratinocytes and skin endothelial cells, the most effective was radiation with $\lambda_{max} = 412$ nm; the photobiological effect decreased with increasing radiation wavelength. Moreover, despite the fact that radiation with $\lambda_{max} = 453$ nm practically corresponds to the absorption maximum of flavins ($\lambda_{max} = 447$ nm), it (in contrast to radiation with $\lambda_{max} = 412, 419, 426$ nm) had practically no toxic effect on cells even with increasing energy dose to $D = 500$ J/cm². At the same time, as can be seen from Fig. 4, radiation with wavelengths of $410, 412, 419$ and 420 nm corresponds to the region of the local minimum in the absorption spectrum of flavins and the region of maximum of the Soret band in the absorption spectrum of endogenous porphyrins, and radiation $\lambda_{max} = 453$ nm practically corresponds to the region of the maximum in the absorption spectrum of flavins and the local minimum in the absorption spectrum of porphyrins. Thus, when studying the effect of blue light on non-cancer cells, the contribution of endogenous porphyrins to the effects of photoinactivation is also beyond doubt.

Finally, comparative studies on fluorescence (Fig. 6) performed with acidic extracts of cancer and normal cells revealed that the concentration of protoporphyrin IX in cancer cells is approximately 2.5 times higher than its concentration in normal cells. In our opinion, the higher concentration of endogenous porphyrins in cancer cells is the reason for their higher sensitivity to blue light compared to normal cells.

Taking together, the results obtained in this work, show: (a) a higher rate of inhibition of the metabolic activity of cells by radiation with wavelength $\lambda = 405$ nm, corresponding to the maximum in the absorption spectrum of protoporphyrin IX, compared to the action of light with $\lambda = 445$ nm, corresponding to the maximum in the absorption spectrum of flavins; (b) a higher rate of inhibition of metabolic activity of HeLa cancer cells by blue light compared to normal BGM cells; (c) higher levels of ROS formation, recorded using the chemiluminescence assay, when a cell suspension is exposed to radiation with $\lambda = 405$ nm compared with $\lambda = 445$ nm; (d) higher levels of ROS formation when exposed suspension of HeLa cells to blue light compared to normal BGM cells; (e) the higher concentration of endogenous porphyrins recorded by the fluorescent method in HeLa cells compared to normal BGM cells allows us to conclude that endogenous porphyrins

localized in cells are capable of playing a leading role in the sensitized formation of ROS when exposed to blue light, affecting metabolic processes in cells, and the higher concentration of endogenous porphyrins in cancer cells is one of the reasons for their higher sensitivity compared to normal cells to blue light.

In connection with the above, a reasonable question arises: why does the contribution of endogenous porphyrins to the effects of sensitized processes in cells become so significant, despite their very low concentration. Indeed, according to Refs. [29, 77], in HeLa cells, the concentration of endogenous porphyrins is ~ 1 nM, while the concentration of flavins in the same cells, according to Refs. [90, 91], is 0.3–1 μ M. Moreover, the ratio of the concentrations of riboflavin, FMN and FAD varies depending on the method of their extraction from cells from 0.40/0.17/0.42 to 0.16/0.096/0.75 [90]. And the quantum yield for the formation of singlet oxygen in aqueous environment at neutral pH value is $\varphi_{\Delta} = 0.54$ for riboflavin, $\varphi_{\Delta} = 0.51$ for FMN, and $\varphi_{\Delta} = 0.07$ for FAD [88]. In addition to singlet oxygen, flavins are also capable of generating superoxide radicals [96] and hydroperoxides [97] upon photoexcitation, although with much less efficacy. As for endogenous porphyrins, the efficacy of singlet oxygen generation upon photoexcitation strongly depends on the state of aggregation and is most significant for the monomeric form. Thus, for monomeric form of protoporphyrin IX $\varphi_{\Delta} = 0.77$ [87], uroporphyrin $\varphi_{\Delta} = 0.80$ [98], coproporphyrin $\varphi_{\Delta} = 0.58$ [99], zinc protoporphyrin IX $\varphi_{\Delta} = 0.91$ [100], zinc-coproporphyrin $\varphi_{\Delta} = 0.54$ [101]. In other words, upon photoexcitation of protoporphyrin, uroporphyrin and zinc protoporphyrin, the efficacy of singlet oxygen generation is approximately 1.5 times higher than the corresponding indicators for riboflavin and FMN, and an order of magnitude higher than for FAD. Since FAD makes up 42–75% of the total flavin content in the cell [94], we can conclude that the quantum yield of singlet oxygen generation is significantly higher when this process is sensitized by porphyrins compared to flavins.

As already noted, in addition to singlet oxygen, the free-base of porphyrins are capable of generating superoxide radicals [102] and hydroperoxides [89]. However, the efficacy of such generation is significantly lower than the efficacy of sensitized formation of singlet oxygen. Consequently, for both porphyrins and flavins, the main intermediate that determines their sensitizing effect is singlet oxygen.

In addition to high efficacy of singlet oxygen formation, another reason for significant contribution of endogenous porphyrins to sensitized damage of cellular structures may be the higher values of the molar extinction coefficient of tetrapyrroles compared to flavins. It is known that, at the maximum of the long-wave absorption band, the molar extinction coefficients are $\varepsilon_{447} = 12.5$ mM $\cdot$ cm $^{-1}$ for riboflavin and FMN, and $\varepsilon_{447} = 11.3$ mM $\cdot$ cm $^{-1}$ for FAD [103]. For endogenous porphyrins in monomeric form (in chloroform), the corresponding values of the molar extinction coefficients at the maximum

of the Soret band are at least an order of magnitude higher: for protoporphyrin IX – $\varepsilon_{408} = 171$ mM $\cdot$ cm $^{-1}$; for coproporphyrin – $\varepsilon_{400} = 180$ mM $\cdot$ cm $^{-1}$; for uroporphyrin – $\varepsilon_{406} = 217$ mM $\cdot$ cm $^{-1}$ [104]. Naturally, such significant differences in the molar extinction coefficients of porphyrins and flavins also contribute to the manifestation of the sensitizing properties of endogenous tetrapyrroles when exposed to blue light.

In addition to above factors that contribute to significant role of endogenous porphyrins in photobiological processes in cells when exposed to blue light, differences in intracellular localization of porphyrins and flavins may play a certain role. As for porphyrins, according to fluorescence microscopy, endogenous protoporphyrin IX, the formation of which in cells is induced by 5-aminolevulinic acid (ALA), is initially localized in mitochondria, and, in this case, it has the most pronounced photodynamic effect [105]. Unlike endogenous protoporphyrin IX, chemically pure exogenous protoporphyrin IX is predominantly distributed in cell membranes. At the same concentrations of endogenous porphyrin in cells and its exogenous analogue, photodynamic damage to cells (both cancer and normal) was significantly higher for the endogenous porphyrin [105]. This result indicates the importance of the region of localization of the sensitizer in the cell for the manifestation of its photodynamic activity.

Regarding the localization of flavins in cells, according to confocal and fluorescence lifetime imaging microscopy [106], endogenous flavins are predominantly localized outside the cell nucleus in mitochondria. Their fluorescence is characterized by a maximum in the region of $\lambda_{max} < 500$ nm, and the fluorescence lifetime is $\tau < 1.4$ ns, which indicates that flavins are predominantly in protein-bound form. In some part of the cells, the fluorescence came from flavins localized inside the cell nuclei, in nucleoli, demonstrating a longer fluorescence lifetime and a red-shifted spectral maximum, indicating the presence of flavins in free form [106]. Somewhat different data from the above were obtained by the authors of Ref. [78]. According to their results, based on the use of electron paramagnetic resonance methods to detect oxyradicals formed in cells upon exposure to blue light, flavin photosensitizers are found predominantly in the cytosol of cells and have a mass of less than 12 kDa. This also indicates a bound form of flavins, probably flavin-protein complexes.

In this regard, it should be noted that when flavins form complexes with proteins, there is reason to believe that the sensitizing effect of flavins will be primarily directed at the protein with which they are in complex [107, 108]. This is due to the short lifetime of singlet oxygen in aquatic biological environments, and, accordingly, the short length of its path from the place of formation, that is, from the sensitizer. In fact, the carrier protein protects cellular structures from sensitized photodamage, which may also be responsible for the reduction in contribution of flavins to cell photodamage upon exposure to blue light. And finally, it is well

known [109] that riboflavin, along with a sensitizing pro-oxidant effect, also has pronounced antioxidant properties. Consequently, upon photoexcitation of flavins with subsequent formation of ROS, flavin molecules can act as ROS quenchers through the mechanism of physical or chemical quenching, including the conversion of reduced riboflavin to the oxidized form [109].

Thus, (a) higher efficacy of singlet oxygen formation sensitized by porphyrins and higher values of their molar extinction coefficients compared to flavins; (b) localization of porphyrins in mitochondria, while a significant proportion of flavins are localized in the cytosol; (c) binding of flavins to proteins that perform a protective function against flavin-sensitized damage to cellular structures; (d) the pronounced antioxidant properties of flavins promote the significant contribution of endogenous porphyrins to the regulatory effects, initiated in cells by exposure to blue light, despite their lower (compared to endogenous flavins) concentration.

It should be noted that the possible role of endogenous porphyrins as acceptors of radiation in the blue spectral region in cells of various types was indicated in a number of works [1, 2, 18, 29, 64, 65, 73–77, 110, 111]. At the same time, in an interesting and informative review [2], it is stated without evidence that the concentration of flavins and porphyrins in cancer cells is low compared to normal cells, and it remains controversial whether they are precisely those photoreceptors that, upon exposure to blue light, suppress cell viability. In addition, according to Ref. [2], due to the presence of various components in cell culture media, it is difficult to determine which compound causes blue light-induced cell death.

A point of view close to the conclusions of Ref. [2] is also shared by the authors of Ref. [18], according to whom the concentration of flavins and porphyrins in cancer cells compared to normal cells is controversial and very few direct or significant comparisons are available. Additionally, due to the variety of cell culture media used, determination of specific chromophore from a cocktail makes it difficult to identify the compound responsible for blue light-specific cell death [18].

We believe that the results obtained in this work convincingly prove the important role of endogenous porphyrins in the implementation of photobiological processes in cells upon exposure to blue light, as well as the importance of higher concentrations of endogenous porphyrins in cancer cells compared to normal ones in initiating higher levels of formation ROS, and as a result - in ensuring a higher sensitivity of cancer cells to the blue light.

At the same time, the results of comparative studies of the effect of optical radiation with wavelengths of $\lambda = 405$ nm and $\lambda = 445$ nm on the metabolic activity of cells (see Fig. 1) and on the integral intensity of chemiluminescence of cell suspension (see Fig. 5), which revealed the participation endogenous porphyrins in sensitized processes in cells, do not at all deny the participation of endogenous flavins in these processes.

As already noted, the absorption coefficient of protoporphyrin IX upon transition from wavelength $\lambda = 405$ nm to $\lambda = 445$ nm decreases by more than 10 times, while the integral intensity of chemiluminescence of cell suspension according to the data in Fig. 5 is reduced by only 19%. The rate of light-induced decrease in the metabolic activity of cells also changes less significantly (as the absorption coefficient) when moving from wavelength of $\lambda = 405$ nm to $\lambda = 445$ nm (Fig. 1). All this indicates the contribution of not only endogenous porphyrins, but also endogenous flavins in the sensitization of cells when exposed to blue light. In this case, the contribution of flavins is most pronounced in the region of maximum of their absorption spectrum, where porphyrins are characterized by minimal absorption. Conversely, the contribution of porphyrins is most pronounced in the region of the maximum absorption band in the Soret band, corresponding to the local minimum of the absorption spectrum of flavins (Fig. 4).

In our opinion, the participation of ROS (singlet oxygen, hydroxyl radicals, hydrogen peroxide, superoxide anion radical, etc.) formed during exposure of cells to blue light is not limited to lipid peroxidation, oxidation of proteins and nucleic acids, inhibition of enzymes and activation of apoptosis, which in ultimately leads to cell death [112]. Indeed, at high energy doses of light exposure, the contribution of these destructive processes initiated by ROS to the decrease in the metabolic activity of cells is significant, which is confirmed by the registration of a large proportion of dead cells [7, 17, 19, 22, 35, 43, 47, 49, 52]. At the same time, it is well known that low concentrations of ROS can act as signaling molecules that can have a regulatory effect on metabolic processes in cells [1, 64–66]. A change in the intracellular concentration of ROS under light can change the redox status of the cell and can be a direct cause of both the activation of proliferative processes in cells (at a relatively low level of ROS) and the inhibition of their proliferation (at an increased concentration of ROS).

Indeed, studies [4, 12, 27, 43, 49, 57], using flow cytometry, have shown that at low energy doses of blue light, a decrease in the metabolic activity of cells is not realized due to their death, since the decrease in metabolic activity is practically not accompanied an increase in the number of necrotic and apoptotic cells. It is believed [4, 12, 27, 43, 49, 57] that the inhibitory effect of light in this case is the result of its influence on the cell cycle, and is explained by the inhibition of the transition from G1 to S-phase and an increase in the duration of the M-phase. Note that, when monitoring cell cycle parameters, the specificity of the response of cancer and normal cells to the blue light is also noted. Inhibition of cell growth not as a result of the death of some cells, but due to prolongation of the stages of the cell cycle, also indicates the regulatory (non-destructive) nature of the action of blue light at relatively low energy doses. At the same time, as the energy dose of light exposure increases, along with a decrease in the metabolic and proliferative

activity of cells, an increase in the number of dead cells is noted, and cell cycle arrest is also initiated.

Another confirmation of the mild regulatory nature of the action of blue light on animal somatic cells (including cancer cells) is the registration of its stimulating effect in a certain dose range: stimulation of DNA and RNA synthesis when exposed to narrow-band blue light with a wavelength of $\lambda_{max} = 404$ and 434 nm at energy dose $D = 1 \text{ mJ/cm}^2$ [93], stimulation of cell proliferation by laser radiation with a wavelength $\lambda = 405$ nm at energy dose of $D = 30 \text{ J/cm}^2$ [11], stimulation of metabolic activity of cells by laser radiation or radiation from LED source with $\lambda_{max} = 405$ nm, at $D = 0.45 \text{ J/cm}^2$ [29]. At the same time, the dose dependence of the biological effect of blue light, according to Refs. [29, 93], is biphasic in nature, which indicates its compliance with the well-known Arndt–Schultz rule [113], according to which, at low doses of exposed radiation, a slight stimulating effect is observed, which increases with increasing energy dose until the maximum is reached; further increase in the dose leads first to a decrease in the stimulating effect to the control level, and then to an inhibitory effect compared to non-irradiated samples, and finally to the death of the biological object. As noted earlier [74, 75], compliance of the dose dependence with the Arndt–Schultz rule indicates the regulatory nature of the action of a physical factor on a biological system, that is, when blue light is exposed to cancer and normal cells, all the signs inherent in the effects of photobiomodulation appear [113].

However, it should be noted that higher levels of ROS production in cancer cells are not the only reason for their greater sensitivity to blue light. Thus, some studies note that the causes of increased sensitivity of cancer cells are excessive accumulation of ROS, as well as deficiency of antioxidants, which leads to oxidative damage to mitochondria [23]. A similar point of view is shared by the authors of Ref. [36], according to whom cancer and normal cells differ in their cellular response to ROS, and cancer cells may be more prone to apoptosis as a result of an imbalance of the intracellular antioxidant system caused by excessive production of ROS.

Thus, the results presented in this paper, compared with the analysis of literature data, provide an answer to the reasons for the higher sensitivity of cancer cells compared to non-cancerous cells to the action of optical radiation in the blue spectral region (both laser and non-laser sources).

5 Conclusions

The studies carried out in this work showed the important role of endogenous porphyrins (free-base and their zinc complexes) in the generation of reactive oxygen species in cells (and, above all, singlet oxygen), which are capable of influencing, by changing the redox state of cells upon absorption of blue light, the metabolic processes occurring in them. It has been shown that the decisive role of porphyrins in the effects of cell

sensitization occurs despite higher, at least two orders of magnitude, concentrations of flavins (riboflavin, FMN, FAD) in cells.

The manifestation of the sensitizing properties of porphyrins in cells is facilitated by: (a) higher efficacy of formation of singlet oxygen sensitized by them and higher values of their molar extinction coefficients compared to flavins; (b) localization of porphyrins in mitochondria, while a significant proportion of flavins are localized in the cytosol; (c) binding of flavins to proteins that perform a protective function against flavin-sensitized damage to cellular structures; (d) pronounced antioxidant properties of flavins, which contributes to the quenching of ROS generated by them during photoexcitation.

The leading role of porphyrins, and not flavins, in the photobiological processes that determine cell metabolism when exposed to blue light is confirmed by a higher rate of inhibition of cell metabolic activity and higher levels of ROS formation, recorded using the chemiluminescence assay, when a cell suspension is exposed to radiation with $\lambda = 405$ nm compared to $\lambda = 445$ nm. In this case, radiation with a wavelength of $\lambda = 405$ nm corresponds to the maximum of the absorption spectrum of protoporphyrin IX and the local minimum of the absorption spectrum of flavins, and radiation with $\lambda = 445$ nm corresponds to the maximum of the absorption spectrum of flavins and the region of local minimum of the absorption spectrum of porphyrins.

For the first time, it has been shown that one of the reasons for the increased sensitivity of cancer cells compared to normal cells to the blue light is the higher concentration of endogenous porphyrin sensitizers, which is confirmed by fluorescent analysis methods. The determining role of porphyrins in the difference in the reactions of cancer and normal cells to the blue light on their suspension is also evidenced by higher levels of ROS formation and a higher rate of light-induced inhibition of the metabolic activity of HeLa cancer cells compared to normal BGM cells.

In addition to differences in the concentrations of endogenous porphyrin sensitizers in cancer and normal cells, the reason for the specificity of their reactions to exposure to blue light may lie in the fact that cancer and normal cells differ in their cellular response to the effects of ROS; and cancer cells may be more prone to apoptosis as a result of an imbalance in the intracellular antioxidant system caused by excess ROS production.

Declarations of Interests

The authors declare no conflict of interest.

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Estimation of Blood Flow Velocity in the Heart of Fish Larvae Using Autocorrelation of Echocardiographic Signals

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Abstract. The paper is devoted to the estimation of blood flow velocity in the heart of a fish larva using a high-frequency ultrasonic scanner with a highly focused single-element ultrasonic transducer. The two-dimensional echocardiographic signal, recorded at a given probe position as a function of the delay of the reflected wave and time, is a superposition of randomly distributed responses reflected from moving blood cells. It is shown that a single value of flow velocity can be estimated by calculating the autocorrelation function of the echocardiographic signal, cropped by a two-dimensional data window. Moreover, for this purpose it is sufficient to calculate only the phase of the autocorrelation function at two adjacent points shifted by sampling intervals. The developed processing algorithm was verified using echocardiographic data measured on sturgeon larva aged 3 days after hatching. An ultrasonic transducer with a central frequency of 75 MHz, a bandwidth of 65%, and an angular aperture of 40° was used in the experiment. The velocity calculated in the ventricle area during one cardiac period varied in the range of ± 10 mm/s depending on the contraction phase.

Keywords: ultrasonic focused transducer; sturgeon larva; M-scan; Doppler frequency shift; *in vivo* measurement.

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1 Introduction

Echocardiography, which provides valuable information about the functioning of the cardiovascular system of living organisms, is widely used for clinical and research purposes [1]. In echocardiography, the spatio-temporal distribution of blood flow can be estimated by sending and receiving ultrasound waves [2, 3]. In modern medical scanners, the Doppler frequency shift of waves reflected by moving blood cells is used to determine the projection of the velocity vector onto the axis of the ultrasonic transducer [4, 5]. Some modern techniques make it possible to determine the velocity vector [6–8]. They are based on the rapid acquisition of two-dimensional scans and tracking the movement of blood cells in the longitudinal and transverse directions. The implementation of these techniques is impossible without the use of multi-element ultrasonic phased arrays. Since the heart of humans and other large living organisms is located deep in the body, the frequency range usually does not exceed 10 MHz, the angular aperture of the

probing ultrasonic beam is narrow, and the spatial resolution is in a millimeter range. Higher resolution has been achieved in ultrasound examination of the heart of small animals such as mice and chickens [9–12]. Recently, high frequency echocardiography has been successfully applied in a wide range of works devoted to adult Zebrafish [13–15]. At the same time, it was shown that sonography of Zebrafish model at the embryonic and larval stages of development can be considered very promising for cardiological research [16–17]. Since the size of the fish heart at early stages of development is in the range of 200–300 μm [18], high frequency single element focused transducers were used in these works to ensure sufficient resolution. Due to short ultrasonic pulses with a central frequency in the range of 50–100 MHz and a wide angular aperture of the focused beam, the transverse and longitudinal resolution of the device was about 20 μm . This value is compatible with the typical size of the blood cells (7 μm) which give a strong stochastic reflected signal. Their velocity usually

does not exceed 10 mm/s, and the Doppler frequency shift in this case is too small for direct measurement in the spectral domain. The generation of echocardiographic signals from the Zebrafish larvae has been partly discussed in our previous work [19].

Typically echocardiographic signals are recorded in the M-scan format $s(t, T)$ [2, 3], where the “fast” time-of-flight t is associated with the propagation of the ultrasonic waves and the “slow” observation time T is associated with the motion of the cardiac tissue. To determine the flow velocity, it is sufficient to measure the delays of ultrasonic echoes reflected from moving blood cells and, knowing the speed of sound in the liquid, calculate their displacement over a certain time interval T [2]. The delay can be obtained by isolating the pronounced echoes from others and using, for instance, the zero-crossing method. This approach is quite straightforward, but very often it is not possible to find a wavelet suitable for the analysis in the area of interest, especially using a fully automated software procedure.

A more advanced method of flow velocity estimation is based on the correlation approach. The relative delay of successive responses can be determined from the time shift of the peak of the one-dimensional cross-correlation function between one-dimensional or two-dimensional windowed data sets [2, 3]. This method has been successfully applied to study blood flow in fish larval hearts [16, 20], however a more reliable result could potentially be achieved using a two-dimensional autocorrelation approach [21].

It is shown that the velocity can be estimated from the phase of the two-dimensional autocorrelation function in the vicinity of its central lobe. This method is based on the assumptions that the velocity of moving cells does not change along the axis of the ultrasound beam and that its angular aperture is narrow. The efficacy of the two-dimensional autocorrelation technique was confirmed in a study of adult Zebrafish with a heart size of 1–2 mm [22]. Since moderate frequencies and angular apertures were used in this work, the assumptions made in developing the signal model should be considered acceptable. For small organism, highly focused high-frequency ultrasonic transducers are used, and the blood

velocity varies with defocus distance, therefore the autocorrelation approach needs to be verified. In this paper, we present an autocorrelation method developed to estimate the blood flow velocity in the heart of fish at the larval stage of development.

2 Experiment

A high-frequency ultrasonic scanner was used to study the fish larval hearts *in vivo* (Fig. 1(a)). The device was equipped with a single element focused ultrasonic transducer with a central frequency of 75 MHz and a bandwidth of 65%. The angular aperture of the transducer was 40° , which provided a lateral resolution of approximately 20 μm . The transducer was excited by short (5 ns) electrical pulses and the signals reflected by the object were converted into digital form after filtration and amplification and stored in the computer. The position of the transducer relative to the object under study was controlled by a precise 3-axis mechanical scanner. To detect the temporal behavior of the heart at each transducer position (x, y) , M-scans $s(t, T)$ were recorded for several seconds. The sampling periods of the digitized M-scans for the variables t and T were $\delta t = 2$ ns and $\delta T = 0.2$ ms, respectively. A more detailed description of the ultrasonic scanner can be found elsewhere [23].

In the experiment, sturgeon larva aged 3 days after hatching (Fig. 1(b)) was placed in an immersion cell filled with water. To immobilize the animal, a solution of trichloroethane methane sulfonate (MS-222) at a concentration of 0.168 mg/L was used for anesthesia. The sagittal plane of the larva was oriented perpendicular to the z -axis of the transducer.

An example of M-scan $s(t, T)$ taken at point O of the heart (Fig. 1(b)) is shown in Fig. 2(a) as a grayscale image. The data are presented for one period of heart rhythm $T_0 = 0.454$ s. Several echo signals can be recognized in the images, the delays of which change with time T . The echoes M_1 , M_2 are generated by the layered tissue of the myocardium, they show the upper and low walls of the heart chamber, respectively.

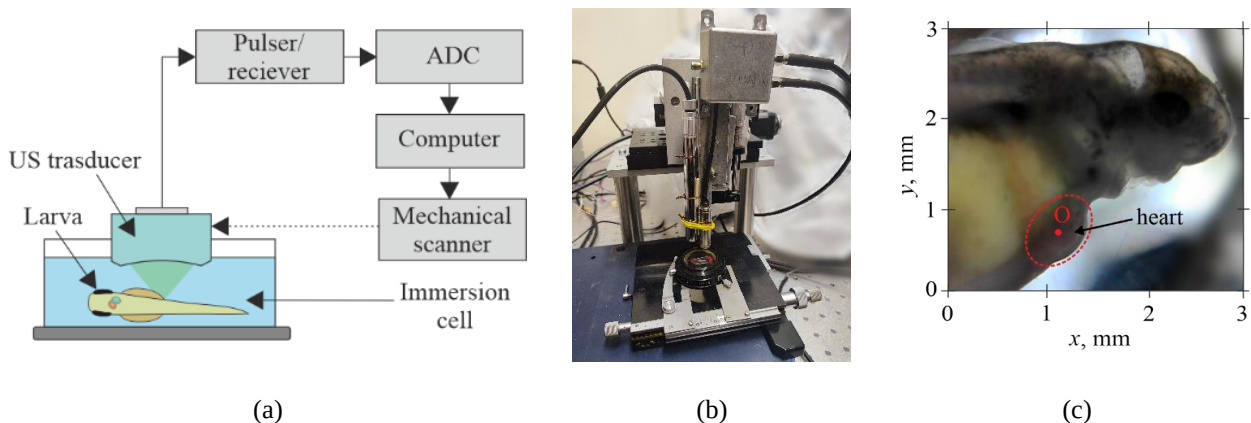


Fig. 1 (a) Scheme and (b) appearance of the experimental setup, (c) optical image of the heart area of the sturgeon larva.

The positions of these echoes change with time T in accordance with the heart contraction. Numerous noise-like B echoes result from scattering of the ultrasound probe wave by moving blood cells. Fig. 2(b) shows an enlarged fragment of this response, indicated in Fig. 2(a) by the yellow rectangle. The local slope of the wavelets B is proportional to the flow velocity V [2]. Indeed, during a time interval ΔT , blood cells, moving in the vertical direction and acquiring a delay Δt , travel a distance of $\Delta z = 0.5C\Delta t$, where $C \approx 1500$ m/s is the sound velocity in the tissue. Therefore, the velocity V can be estimated using a simple equation:

$$V = C\Delta t(2\Delta T)^{-1}. \quad (1)$$

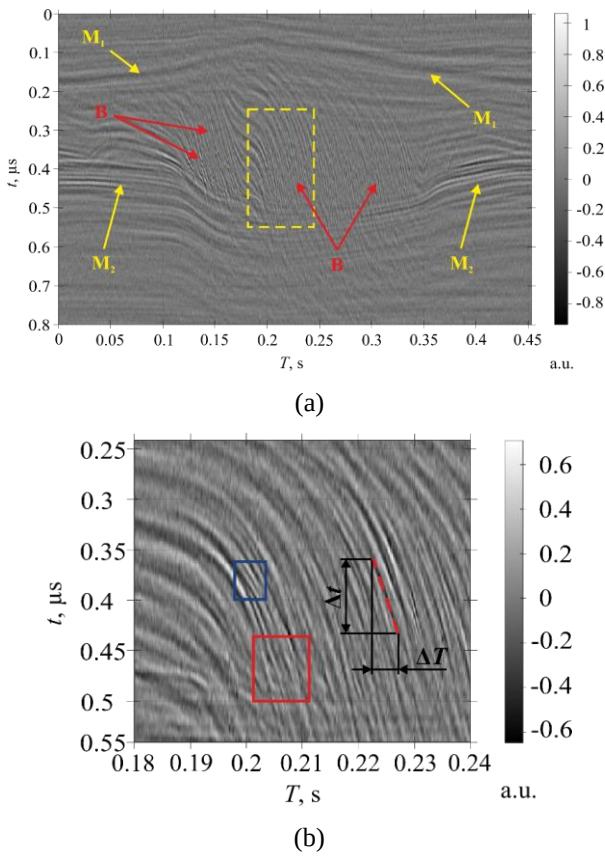


Fig. 2 (a) M-scan $s(t, T)$ recorded at the ventricle of the sturgeon larva. (b) Enlarged fragment of the scan marked in the (a) image with a yellow rectangle. B, M_1 , M_2 are echo signals generated by blood cells, the upper and low walls of the heart chamber, respectively; Δt is the delay acquired by blood cells when moving along the red dotted line during the time interval ΔT .

The size of the blood components, mainly red blood cells, is about 5–10 μm , therefore there are approximately 10 reflectors in the resolution volume. The interference of the echoes from these randomly distributed cells forms a stochastic response B. Since the orientation and position of the reflectors change over time, the coherence duration is limited. Therefore, it is often impossible to find a wavelet that maintains integrity over the interval ΔT and to reliably estimate the delay Δt .

A more robust estimate of the flow velocity can be obtained using a two-dimensional autocorrelation approach.

3 Theoretical Model of the Signal

To find the responses from the blood flow received by the ultrasonic transducer, the following assumptions can be accepted. The blood elements can be considered as point reflectors; their number is large within the focal volume. In the two-dimensional autocorrelation approach proposed in Ref. [21], it was assumed that the ultrasonic beam is wide, without a clear focal zone; and the spatial distribution of the velocity is axially symmetric and does not change along the axis. In the case of the tightly focused beam (Fig. 3), the signal $h(t, \bar{r}, z)$ received from the reflector depends on time t and position $(\bar{r}, z)$. The reflectors move at a velocity of $\bar{V}_b = (V, \bar{V}_r)$, where V and $\bar{V}_r$ are the vertical and radial components of the velocity vector. In a layer with a thickness of $2\Delta z$ and a center $z = z_a$, the velocity remains approximately constant during the observation time interval $|T| \leq \Delta T$. Since the velocity of the blood cells is much less than the velocity of ultrasound waves in the tissue C , the response from the i -th moving particle can be written as follows:

$$s_i(t, T) = a_i h(t, \bar{r}_{0i} + \bar{V}_r T, z - z_{0i} - VT), \quad (2)$$

where a_i is the reflection coefficient of the i -th particle, and $(\bar{r}_{0i}, z_{0i})$ is its initial position at the moment $T=0$.

The output signal is a sum of wavelets given in Eq. (2) whose response are in the time-of-flight interval $|t - t_a| \leq \Delta t$, where $t_a = 2z_a C^{-1}$ and $\Delta t = 2\Delta z C^{-1}$.

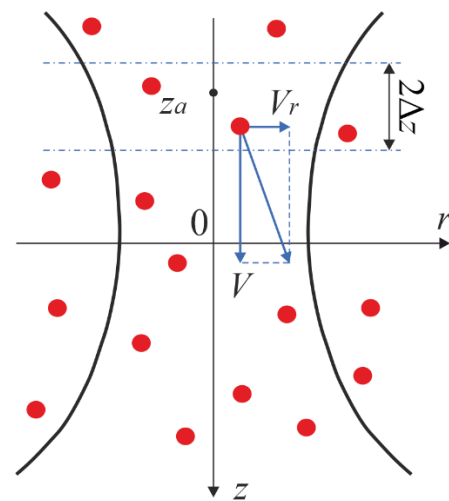


Fig. 3 The focal area of an ultrasonic beam containing an ensemble of moving particles. V and V_r are the vertical and radial components of the velocity; z_a and $2\Delta z$ are the position and the thickness of the layer, respectively.

The wave fronts of the ultrasonic beam in the vicinity of the focus are approximately parallel to the focal plane [24], therefore motion of the particle along vertical axis z causes a delay of the reflected signal $2VTC^{-1}$. On the contrary, lateral motion does not change the signal delay, but it can change the amplitude of the signal. Since the delay is the same for all particles in the layer, the sum of their responses can be written as a two-dimensional convolution:

$$s_0(t, T) = \left[\sum_i a_i h_r \left(t, \bar{r}_{0i} + \bar{V}_r T, z - z_{0i} \right) \right] * \delta \left(t - \frac{2VT}{C} \right), \quad (3)$$

where $\delta()$ is the Dirac delta function. The function h_r shows the changes in the amplitude and delay of the temporal response $h(t)$ of the i -th reflector caused by its lateral displacement. The amplitude of the function h_r decreases smoothly as z_a deviates from the focal plane $z = 0$. The lateral displacement also causes amplitude variations determined by the profile of the ultrasonic beam. The duration of this response is limited by the travel time of the particle across the beam $\delta r/V_r$. Typically, the z -axis is oriented approximately along the blood flow so that $V_r \ll V$, and the particle remains inside the ultrasonic beam for the observation time interval ΔT . Summing the responses over the entire ensemble of the reflectors yields a stochastic function $g(t, T)$, and the output signal can be represented within the intervals Δt , ΔT as follows:

$$s(t, T) = \left[g(t, T) * \delta \left(t - \frac{2VT}{C} \right) \right] w_t(t) w_T(T), \quad (4)$$

where the window functions $w_T(T)$ and $w_t(t)$ cut out the data region intended for processing. In the observation intervals ΔT , Δt , the signal behavior is determined by the liner delay factor. This relationship given in Eq. (4) is equivalent to the signal model developed under other assumptions [18]. Using the methodology presented in this work, the following results can be obtained. The autocorrelation function $A(t, T)$ of a signal $s(t, T)$ can be found by taking the inverse Fourier transform of the signal power spectrum [25]:

$$A(t, T) = F^{-1} \{ S(\omega, \Omega) S^*(\omega, \Omega) \}, \quad (5)$$

where the symbol $*$ denotes the complex conjugation operation, and the frequencies ω , Ω are associated with the time variables t , T . The spectrum of the signal (Eq. 5) is equal to:

$$S(\omega, \Omega) = \left[G(\omega, \Omega) \cdot \delta \left(\Omega - \frac{2V}{C} \omega \right) \right] * W_\omega(\omega) * W_\Omega(\Omega), \quad (6)$$

where G , W_ω , W_Ω are spectra of g , w_t , w_T , respectively. In the case of infinite window functions w_t , w_T the convolution with W_ω , W_Ω in Eq. (6) can be omitted, and the spectrum $S(\omega, \Omega)$ is equal to 0 everywhere in the (ω, Ω) domain, except for the line $\Omega = 2VC^{-1}\omega$. Therefore, the variable Ω can be considered as the Doppler frequency shift obtained by the component of the probing wave with frequency ω due to reflection from the moving scatterer. The slope of this line depends on the velocity V , similarly the orientation of the components in the signal $s(t, T)$ and in its autocorrelation function $A(t, T)$ also depends on V . To estimate this slope, one can use the partial derivatives of the autocorrelation function with respect to the variables t and T .

In the case of infinite data windows, based on the definition of the Fourier integral and the properties of the delta function, the value of the derivative with respect to t of the autocorrelation function at the center $(0, 0)$ is equal to:

$$\begin{aligned} r_t &= \frac{\partial A(t, T)}{\partial t} \Big|_{t=0, T=0} = \\ &= (2\pi)^{-2} \int_{-\infty}^{\infty} (-i\omega) \left[\left[G \left(\omega, \frac{2V}{C} \omega \right) \right] \right]^2 d\omega. \end{aligned} \quad (7)$$

The partial derivative with respect to T can be obtained in a similar way:

$$r_T = \frac{\partial A(t, T)}{\partial T} \Big|_{t=0, T=0} = \frac{2V}{C} r_t. \quad (8)$$

Eq. (8) shows that the ratio of the coefficients r_T and r_t is proportional to the velocity V . In the case of finite data windows, the spectrum $S(\omega, \Omega)$ is smoothed due to the convolution given in Eq. (6), and Eq. (7) is invalid. However, it has been shown [21] that the coefficients r_T and r_t are the averaged values of the frequencies ω and Ω , respectively, and the relation given in Eq. (8) is applicable for estimating the velocity V .

It should be noted that digital calculation of derivatives of noisy experimental data may be unstable. However, since the amplitude and phase $\varphi(t, T)$ of the autocorrelation function are even and odd functions, respectively, the following approximation is valid:

$$\begin{aligned} \frac{\partial A(t, T)}{\partial t} \Big|_{t=0, T=0} &= iA(0, 0) \frac{\partial \varphi(t, T)}{\partial t} \Big|_{t=0, T=0} \approx \\ &\approx iA(0, 0) \frac{\varphi(\delta t, 0)}{\delta t}, \end{aligned} \quad (9)$$

where δt is the sampling period of the time-of-flight t . Using a similar relationship for the derivative with respect to the time T , Eq. (8) can be rewritten:

$$V = C \frac{\varphi(0, \delta T) \cdot \delta t}{2\varphi(\delta t, 0) \cdot \delta T}, \quad (10)$$

where δT is the sampling period of the time T . Thus to estimate the velocity V at a specific point in the (t, T) domain it is sufficient to calculate the phase of the

autocorrelation function at two adjacent points shifted by intervals $\delta t, \delta T$.

4 Data Processing Algorithm

The experimental digital M-scan is a sampled version of the real valued signal presented as an n -by- m array $s_{nm} = s(n \cdot \delta t, m \cdot \delta T)$, where n and m are indexes related to the variables t and T , respectively. Before calculation of the autocorrelation function, the input data are converted to analytic signal $r_{nm} = s_{nm} + iH\{s_{nm}\}$, where i is the imaginary unit, and $H\{\}$ is the Hilbert transform with respect to n . To find the values of the autocorrelation function $A_{nm}^{(\delta t, 0)}$ and $A_{nm}^{(0, \delta T)}$ at the lags $(\delta t, 0)$ and $(0, \delta T)$, respectively, first the element-wise products of the array r_{nm} and its shifted and complex conjugate copies are calculated:

$$\begin{aligned} D_{nm}^{(10)} &= r_{nm} \cdot r_{(n+1)m}^*, \\ D_{nm}^{(01)} &= r_{nm} \cdot r_{n(m+1)}^*. \end{aligned} \quad (11)$$

The signal power is calculated in a similar way:

$$D_{nm}^{(00)} = r_{nm} \cdot r_{nm}^*. \quad (12)$$

To find the values of the autocorrelation function, it is necessary to average the products given in Eq. (11) in sliding two-dimensional windows. This operation can be implemented using a low-pass two-dimensional finite impulse response filter w_{nm} :

$$\begin{aligned} A_{nm}^{(\delta t, 0)} &= D_{nm}^{(10)} * w_{nm}, \\ A_{nm}^{(0, \delta T)} &= D_{nm}^{(01)} * w_{nm}, \\ A_{nm}^{(0, 0)} &= D_{nm}^{(00)} * w_{nm}. \end{aligned} \quad (13)$$

The spatio-temporal distribution of the velocity V_{nm} can be obtained using the discrete version of Eq. (10):

$$V_{nm} = \frac{C\delta t \cdot \varphi(A_{nm}^{(0, \delta T)})}{2\delta T \cdot \varphi(A_{nm}^{(\delta t, 0)})}. \quad (14)$$

The proposed algorithm is absolutely robust, since the output values V_{nm} can be calculated for all n and m for any input data s_{nm} . However, in many areas of the (n, m) domain there are no reflections from blood cells, and the signal-to-noise ratio is low. To exclude false results, the maximum value of the autocorrelation function $A_{nm}^{(0, 0)}$ is compared with a certain threshold A_0 . To mask these unreliable values the output is reset to 0: $V_{nm} = 0$, if $A_{nm}^{(0, 0)} < A_0$.

It should be noted that the algorithm is computationally efficient. Indeed, it is sufficient to calculate only 3 values of the autocorrelation function

given in Eq. (13) for each output value V_{nm} . In the case of a boxcar window w_{nm} , basically only $N_f \cdot M_f$ sum operations are required, where N_f, M_f are the window sizes. In contrast, in the one-dimensional cross-correlation algorithm, it is necessary to calculate the correlation function and find its peak for each point in the (t, T) domain.

The implementation of the two-dimensional autocorrelation approach presented in the works [21, 22] is based on demodulation of received signals and digital processing of quadrature components. In the present work, the bandwidth of the signal is wide, so the analog demodulation is ineffective. The velocity estimation accuracy is expected to remain the same since both algorithms are mathematically equivalent. However, additional threshold processing provides greater stability of results for small larval hearts, where it is difficult to locate cardiac chamber boundaries and exclude areas with low signal-to-noise ratio.

5 Results and Discussion

The estimated spatio-temporal distribution of blood flow velocity V is shown in Fig. 4. The calculation was performed using the input M-scan data shown in Fig. 2. The velocity value is color-coded according to the color scale shown on the right side of the picture. The threshold was equal to $A_0 = 0.36 \cdot A_{max}$, where A_{max} is the global maximum of the autocorrelation function $A_{nm}^{(0, 0)}$. The window sizes were set equal to $N_f = 16$ and $M_f = 20$. These numbers correspond to the window (32 ns) by (4 ms) indicated in the (t, T) domain by a blue rectangular (Fig. 2(b)). This window is small enough compared to the time range t (≈ 600 ns) and the heart rhythm period $T_0 = 0.454$ s to correctly show the change in velocity V depending on the vertical position and the phase of the heart contraction.

At two intervals of the time $T = (0.12-0.15)$ s and $T = (0.2-0.24)$ s (Fig. 4) the blood flow is directed downwards, where the velocity reaches a peak value of $V \approx -15$ mm/s. At the intervals $T = (0-0.12)$ s, $(0.15-0.2)$ s and $(0.41-0.45)$ s, the movement of blood cells is slowed down. During a short time interval $T = (0.35-0.37)$ s and in the time-of-flight interval $t = (0.38-0.41)$ μ s, a strong upward flow is observed with a peak velocity of $+15$ mm/s. Outside the cardiac chamber, where the time-of-flight $t < 0.15$ μ s or $t > 0.55$ μ s, the signal is weak, the autocorrelation function is less than the threshold A_0 , and the output was set to 0. Overall, the behavior of the calculated blood flow velocity is in good agreement with the original M-scan (Fig. 2).

The blue graph in Fig. 5 shows the calculated velocity $V(T)$ taken at time $t = 0.385$ μ s along the red dashed line (Fig. 4). The calculation was repeated using the 2D window sizes increased from $N_f \cdot M_f = 16 \cdot 20$ to $N_f \cdot M_f = 32 \cdot 40$ gives (red graph). These windows, superimposed on the measured data $s(t, T)$ are presented in Fig. 2(b) as blue and red rectangles, respectively, to illustrate their relative sizes. It can be concluded that a

larger window decreases the level of fluctuations, but also reduces the resolution due to data smoothing.

The velocity $V(T)$ was also estimated by directly applying Eq. (1) to the raw data $s(t, T)$. Measurement of Δt and ΔT was not possible in all points in the (t, T) domain due to the stochastic nature of the signal. Successfully obtained velocity estimates are presented in Fig. 5 as green crosses. There is good agreement between these values and the results of the autocorrelation approach which confirms correctness of the proposed algorithm.

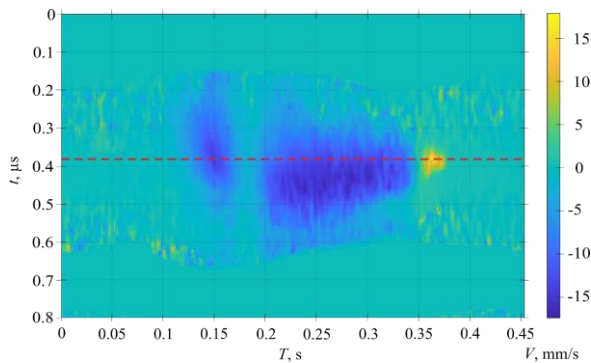


Fig. 4 The velocity $V(t, T)$ calculated using M-scan shown in Fig. 2.

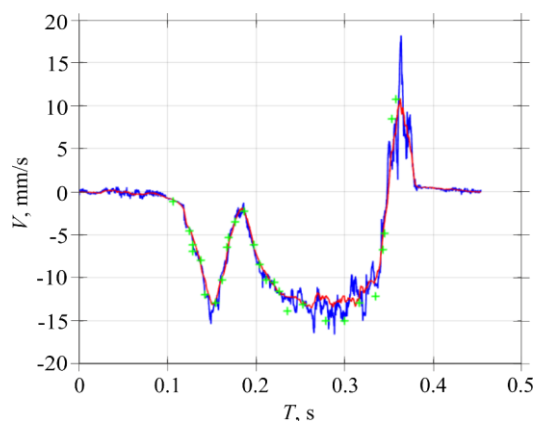


Fig. 5 Velocity $V(t_0, T)$, $t_0 = 0.385 \mu\text{s}$. The window sizes are $32 \text{ ns} \times 4 \text{ ms}$ and $64 \text{ ns} \times 8 \text{ ms}$ (blue and red graphs, respectively). Velocity V estimated using Eq. (1) (green crosses).

6 Conclusion

In this work, an algorithm for processing high-frequency echocardiographic signals recorded using a focused single-element ultrasonic transducer is developed. The algorithm can be used to estimate the spatiotemporal distribution of blood flow velocity in the hearts of small animals, such as fish larvae. To obtain sufficient robustness of the estimation, it is based on two-dimensional autocorrelation analysis of stochastic echocardiographic signals. It is shown that to obtain the value of velocity at a specific point of the heart and for a specific moment of the cardiac period, it is sufficient to calculate only two values of the autocorrelation function. The data used for the calculation are cropped by a local two-dimensional window. The window size is a trade-off between resolution and estimation accuracy. The algorithm is absolutely robust, but it is possible to obtain incorrect values outside the cardiac chambers where the signal to-noise ratio is low. To avoid this, the output value is excluded if the maximum of the autocorrelation function is less than a certain threshold. The developed technique is verified using echocardiographic signals measured *in vivo* in sturgeon larvae. However, it can also be applied to other species with small cardiovascular systems that require the use of a single element focused transducer to obtain high spatial resolution.

All procedures were approved by the Bioethics Committee of the Scientific and Technological Centre of Unique Instrumentation of the Russian Academy of Sciences (STC UI RAS), protocol #1/25, dated 24 February 2025.

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Disclosures

All authors declare that there is no conflict of interest in this paper.

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NIR Imaging: Development of Digital Image Processing Algorithm for Vein Contrast Enhancement

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Abstract. Modern medical laboratory diagnostic methods often require venipuncture, which can be challenging when blood vessels are not visible to the naked eye. This potentially leads to errors in blood sampling and misinterpretation of test results. Optical methods in medicine, specifically, vein visualization in the near-infrared (NIR) range, are actively being developed. However, current methods face several limitations, including shallow imaging depth and low contrast. Additionally, digital image processing algorithms used in these methods are imperfect. These algorithms often have low stability and performance. In this study, an efficient and robust algorithm for digital image processing has been developed to enhance vein visualization. The proposed algorithm outperforms existing methods in terms of the effectiveness of separating pixels corresponding to veins from those associated with surrounding tissues. The algorithm is based on efficient FFT-based operations.

Keywords: vein viewer; OpenCV; convolution; fast Fourier transform; Gaussian filter; ACE; Wallis filter; contrast enhancement.

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1 Introduction

1.1 Background and Related Works

Vein viewer is a medical device used to obtain a pattern of the location of human superficial veins. The operation principle is based on the interaction of IR radiation with biological tissues (Fig. 1). The IR light, emitted in the wavelength range of 700–950 nm, passes through a diffuser. Then it penetrates the skin, illuminating a specific area of the patient's body. Hemoglobin in the blood absorbs this radiation significantly stronger than the surrounding biological tissues, which predominantly reflect the light. This differential absorption and reflection create a contrast between the blood vessels and the surrounding tissues, enabling clear visualization of the venous structure.

The reflected light is captured by a detector sensitive to the IR range, after passing through an optical filter that blocks visible light while allowing infrared light to pass through. This process generates an image that includes both the skin surface and a detailed pattern of the underlying blood vessels. The acquired image undergoes digital processing to

enhance contrast. Then it is displayed on the device's LCD screen.

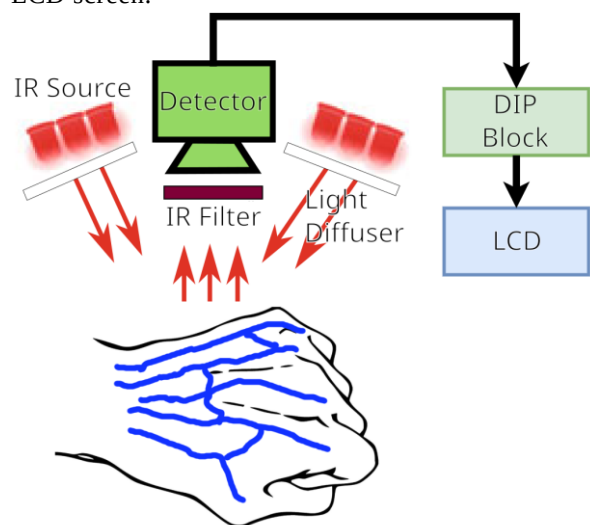


Fig. 1 Vein viewer: the main principle of method.

In medical practice, a vein viewer is a useful tool primarily used for facilitating venipuncture and

supporting treatment planning. This device enhances the visibility of veins in commonly accessed areas for procedures, such as the forearm, hand, lower leg, and foot. These are common sites for catheter insertion, venipuncture, and other leg vein-related interventions, such as sclerotherapy and phlebectomy [1–3]. Beyond its clinical applications, the vein viewer also serves as an important training aid for nurses, helping them to master venous access techniques. It also aids in identifying dilated veins in patients with varicose veins, providing critical information for diagnosis and treatment.

The main limitations of near-infrared vein visualization include reduced vein visibility in certain conditions, such as skin abnormalities, dark skin tones, or excessive body weight. Also, the method is constrained by its limited imaging depth, which typically does not exceed 5 mm [1].

Thus, when diagnosing deep vein disorders, alternative techniques such as phlebography or Doppler ultrasound should be employed. However, for rapid assessment of the location of superficial veins – especially when veins are not visible to the naked eye – small, portable vein-viewing devices are preferred for venous access. NIR Vein viewer can minimize the risk of inappropriate punctures and ensure the safety of the procedure for the patient.

At present, the field of vein visualization remains insufficiently explored. Existing devices lack adequate imaging depth and lack a unified standard for device parameters. Variations exist in optical configurations, as well as in approaches to processing the acquired images [2–12]. A standardized methodology for imaging the human venous system has not yet been developed. This highlights the necessity for further research aimed at optimizing the parameters of vein visualization techniques.

The image obtained from the vein viewer in the NIR range must be digitally processed after its registration and conversion into digital form. Digital processing in the context of vein visualization addresses several tasks: noise and artifact suppression, removal of unwanted components of biological tissues (such as hair, skin defects, and background), enhancement of vein contrast relative to the skin surface, and feature segmentation for constructing a vein pattern. Due to the specific requirements of vein visualization devices, image processing must be performed in real time, which significantly complicates the development of an optimal processing algorithm.

It is important to note that the issue of image processing in the context of vein visualization remains insufficiently explored. At present, no comprehensive studies dedicated exclusively to image processing challenges in vein visualization have been found in the literature. Existing publications on vein imaging prioritize hardware, frequently neglecting image contrast enhancement procedure, with only a brief description of the methods used, or not considered at all. This highlights the need for more comprehensive investigation into this

area to improve the quality and informativeness of the resulting images.

Existing researches efforts utilize approaches aimed at enhancing contrast (e.g., adaptive histogram equalization), suppressing salt-and-pepper noise (median filter), and removing background artifacts.

A. B. Abd Rahman et al. (2022) [3] describe an image processing approach that includes background removal, contrast equalization, and image smoothing. At the first stage, the GrabCut segmentation method is used to remove the background and define the boundaries of the region of interest (ROI). Then, the contrast-limited adaptive histogram equalization (CLAHE) method is applied to the ROI to enhance image contrast. Next, the Hessian segmentation method is applied to separate the vein structure from surrounding noise. This method calculates the probability of a pixel being part of a vessel. Then, median filtering is performed to suppress noise, and the inferno colormap from the Python data visualization library matplotlib is applied to the image. The use of this colormap improves human perception of the image. The algorithm gives a significant increase in the contrast of the veins relative to the skin surface. However, even when disregarding the ROI detection methods, the authors utilize computationally demanding algorithms for filtering, such as median filtering and the Hessian-based segmentation method. Furthermore, the effectiveness of the median filter is highly dependent on the window size, which ideally should be adaptively selected for each image. This requirement may impact the algorithm's robustness when dealing with variations in the capturing angle or perspective.

G. VT (2021) [4] applies a median filter to remove noise, adaptive histogram equalization to enhance contrast, binary segmentation to identify vein regions, and morphological processing to smooth the image and eliminate unwanted areas during vein visualization. The author does not provide a comprehensive mathematical description of the morphological operations used in the algorithm. While real-time operation is claimed, no evaluation of the algorithm's efficiency is presented, and the technical specifications of the equipment used are not provided. Furthermore, the methods employed are not adaptive, which may reduce the reliability of the algorithm's results. In certain cases, this limitation may necessitate manual parameter adjustment by the device operator during operation. A similar approach is proposed in the earlier studies [5, 6].

K. Ahmed et al. (2018) [7] developed an image processing algorithm that begins with the image smoothing stage. In the next step, background detection is performed to remove it and extract the ROI. After a series of transformations aimed at removing artifacts, CLAHE is applied to the extracted region to enhance image contrast. The primary method for enhancing image quality, apart from the removal of extraneous objects, is the CLAHE technique. Although it moderately improves the visibility of veins against the skin surface, the achieved effect is insufficient for the reliable extraction of fine details. A similar approach, utilizing different

noise removal techniques followed by the sequential application of CLAHE, is demonstrated in Ref. [8].

M. Francis et al. (2016) [9] propose the sequential application of a median filter and a Gaussian filter to remove “salt-and-pepper” noise and smooth the image, respectively. Next, CLAHE is performed to enhance image contrast. Then, a Gabor filter is applied to improve the visibility of vein edges. Vein extraction is carried out using the Otsu thresholding algorithm. Then, an erosion operation is performed to construct the vein pattern. Then the ROI is extracted. The algorithm significantly improves image quality; however, the use of the Gabor filter and median filter introduces high computational complexity, making real-time implementation challenging on low-cost hardware.

D. Kim et al. (2017) [10] begin the image processing by extracting the ROI, followed by the application of their custom histogram equalization method. Next, the Frangi filter is applied, and segmentation is performed using an adaptive thresholding method. The authors then employ a “skeletonization” technique, which transforms the vein pattern into thin line-like structures. The algorithm effectively improves the quality of vein visualization. However, the application of the Frangi filter is characterized by high computational complexity, which may limit its applicability for real-time image processing.

N. Sharma and M. Hafeeda (2020) [11] sequentially apply CLAHE and homomorphic filtering, which suppresses low-frequency components and enhances high-frequency components of the image. Subsequently, logarithmic transformation of the image is performed. The homomorphic filtering technique is not clearly explained by authors and does not significantly enhance image contrast.

H. Ozkan et al. (2023) [12] utilize two parallel combinations of filters. In the first case, CLAHE, a median filter, and a bilateral filter are applied sequentially to the image. In the second case, the order of the smoothing filters is altered: CLAHE, followed by a bilateral filter, and then a median filter. According to the authors, this approach enables better vein enhancement in multispectral visualization. The disadvantage of the algorithm is the high computational complexity of the bilateral filter.

M. Jadhav and P. Nerkar (2015) [13] address the problem of biometric authentication, developing a method for finger vein pattern recognition based on field-programmable gate array (FPGA) device. During image processing, a fast algorithm is used, which includes a Gaussian smoothing filter followed by the Canny algorithm for edge detection. For classification, the authors employ several different feature extraction methods. The proposed digital image processing method does not allow for the estimation of vein thickness. This excludes the use of the algorithm for medical purposes.

S. Crisan et al. (2007) [14] used edge detection methods, followed by image binarization. The authors achieved a satisfactory visualization result; however,

they noted the difficulty in selecting the threshold for binarization.

In studies [15–19] on the topic of visualization, the enhancement of image quality through digital processing is either not addressed or discussed only superficially.

Thus, it can be concluded that CLAHE is often used in conjunction with other methods for the processing of NIR images for vein visualization. This method is applied to enhance contrast. Various smoothing techniques are also utilized, particularly the median filter. Special algorithms are employed for edge enhancement. Additionally, background removal and ROI determination methods are actively applied.

In several studies, the CLAHE method is applied to process infrared vein images by performing local histogram equalization to enhance contrast. However, the task of vein visualization requires not only improving brightness distribution but also accurately delineating vessel boundaries. Despite this, in some algorithms, the application of a smoothing filter following CLAHE is considered sufficient. This approach may be inadequate for reliable vein segmentation due to the lack of stages specifically aimed at enhancing sharpness and suppressing noise in low-contrast regions.

As existing studies indicate, the approach to digital image processing is not directly tied to the optical configuration of the device. In other words, the operation of image processing algorithms, in most cases, does not depend on the light sources, filters, or detectors used. Regardless of the device’s configuration, the same methods can be applied for digital image processing. This is because these algorithms operate at the pixel level and their intensity, independent of the characteristics of light sources or filters.

Nevertheless, the effectiveness of these algorithms indirectly depends on the quality of the original image. Image quality includes light distribution, background presence, and noise levels. These factors, in turn, may be influenced by the optical design of the device.

In several algorithms, binarization methods are employed to effectively separate veins from surrounding biotissues. However, these methods also have limitations in terms of adaptability. For instance, the application of global binarization requires threshold adjustment for each specific situation, as factors such as the imaging angle or lighting conditions may vary. These variations directly affect the image histogram and pixel distribution. The Otsu method, for example, has an inherent limitation: it performs optimally when the image histogram exhibits a bimodal distribution. Under conditions of unstable lighting and non-uniform background - which are typical for infrared vein images - the Otsu method may lead to false positives (e.g., inclusion of noisy regions) or the omission of thin vessels. Consequently, the use of binarization may complicate the practical application of the device in real-world medical settings. Moreover, adaptive binarization methods demand significantly higher computational resources.

In the reviewed studies, image processing methods characterized by high computational complexity are employed. The use of algorithms such as the Gabor filter, bilateral filter, and median filtering is demonstrated. These algorithms require significant computational resources, making them impractical for use on devices with limited performance, such as portable vein viewers. In the real-world medical practice, when image processing speed is critical, such delays can be unacceptable. This highlights the need to develop a more efficient image processing method, for example, based on the fast Fourier transform (FFT), which would combine high speed with effective visualization.

Thus, the primary objective of this study is to develop an efficient algorithm for digital processing of images obtained during vein visualization in the NIR spectrum. The algorithm must be adaptive and robust to variations in image capturing conditions. Additionally, it should be based on fast operations, ensuring low computational complexity.

This study addresses the problem of digital image processing for the visualization of venous structures. The research focuses on solving two key tasks: enhancing the contrast of the venous pattern relative to surrounding biological tissues and evaluating the effectiveness of digital filtering results. The work encompasses a comprehensive analysis of existing image processing approaches, the development of a novel algorithm to address the contrast enhancement task, and the quantitative evaluation of the obtained results.

1.2 Specifics of Image Registration in Venous Visualization

Specialized devices known as vein viewers are used to capture digital images of the human venous pattern. The quality of the resulting image is significantly influenced by the optical configuration of the device, which is determined by several key factors. These include: the spectral range of the detected light radiation, the type of light source (LED or laser diode), the shape of the light spot, the sensitivity and resolution of the detector, the characteristics of optical filters and diffusers.

It is known that the maximum penetration depth of light into biological tissues (up to 5 mm) is achieved in the near-infrared range (700–950 nm), whereas the minimum penetration depth occurs in the ultraviolet range (200–400 nm). The penetration depth is also influenced by the characteristics of the radiation source's directional pattern and the width of the light spot. Studies have demonstrated that increasing the width of the incident light spot from 1 mm to 5 mm significantly enhances the light penetration depth into biological tissues. However, starting from a spot width of 10 mm, further increases in width do not affect the penetration depth [20]. The authors attribute this phenomenon to the scattering of narrow light beams caused by the inhomogeneity of the refractive index of biological tissues. Also, the power of the radiation has a minor impact on the penetration depth, as reported in study [21].

In commercial vein viewers, both light-emitting diodes (LEDs) and laser diodes may be used as sources of light radiation. LED-based devices are simpler from a circuit design perspective, with low requirements for the driver circuitry, cooling systems, and calibration processes. However, laser-diode-based devices offer higher result reproducibility. Also, due to their ability to operate within a narrow spectral range and the use of optical filters, laser-based devices can minimize the impact of unwanted spectral components on image quality.

Optical filters must absorb visible light radiation and transmit infrared radiation in the range of 700 to 950 nm, as determined by the specific optical design of the device. The removal of background light in the visible spectrum provides greater contrast, as the radiation reflected from the upper layers of the skin does not reach the detector.

Optical diffuser is not an essential component of a vein viewer; however, its use ensures more uniform and even distribution of light radiation across the surface of biological tissue, thereby preventing overexposure in certain areas of the image. Both the geometric parameters of the diffuser and the material properties are important in this context. Thus, polycarbonate (PC) and polystyrene (PS) plastics show excellent optical properties and can be effectively used as diffusers [22, 23].

The key optical parameters of detectors that affect image quality include the type of sensor array, resolution, and spectral sensitivity. CMOS arrays generally have smaller dimensions and lower cost compared to CCD arrays. However, the architecture of CCD arrays provides higher image quality due to their superior light sensitivity and better signal-to-noise ratio (SNR). The spectral sensitivity of the array determines the range and intensity of light waves of different wavelengths detected by the sensor, which directly impacts color reproduction and overall image quality. Higher spectral sensitivity in the near-infrared (NIR) range allows for a stronger useful signal to be captured at the same exposure time. This, in turn, reduces the relative contribution of noise from the array's amplifiers in the image, thereby increasing the SNR. As a result, the array achieves high speed performance and, consequently, a higher camera frame rate.

2 Materials and Methods

The following steps were carried out during the experiment. A dataset was collected for conducting the experiment. An algorithm for digital image processing was developed using Python and OpenCV. Optimal working parameters of the algorithm were determined to achieve visually satisfactory results. An approach for evaluating the results was proposed.

The developed algorithm is characterized by the use of faster digital processing methods compared to the algorithms in the reviewed articles, which potentially allows for processing on devices with low computational power.

Most existing studies lack a clear methodology for evaluating digital filtering results. When evaluations are

performed [7, 11, 12], researchers focus primarily on vessel segmentation accuracy but often neglect the impact of background (surrounding biotissues) on image quality.

We have developed a novel approach that introduces additional metrics to analyze how the background affects vein contrast and edge sharpness. By incorporating these metrics, our method significantly enhances the objectivity of result evaluation.

2.1 Dataset Compilation

The Forearm Veins dataset, consisting of 100 near-infrared (NIR) images of human forearms, was obtained through the Kaggle machine learning competition platform [24]. The creators of the dataset have not provided information about the conditions under which the data was collected.

The dataset includes forearm images with various challenges, such as poorly visible veins, tattoos, bracers, and excessive hairiness. 70 of 100 images are accompanied by vein mask annotations. Several sample

images from the dataset are shown in Fig. 2, and examples of the corresponding vein masks are presented in Fig. 3.

2.2 Digital Image Processing Algorithm Development

As part of the experimental research, a comparative analysis was conducted to evaluate the effectiveness of applying the CLAHE method to the original dataset versus our custom algorithm effectiveness. We developed an algorithm that incorporates sequential application of high-frequency filtering and contrast enhancement filters.

To develop the algorithm, the Forearm Veins dataset properties were analyzed. Fig. 4 presents histograms of five images, illustrating typical parameter variations. These images were selected for their ability to demonstrate the dataset's wide parameter variability, enabling a concise visual assessment of the algorithm's robustness.

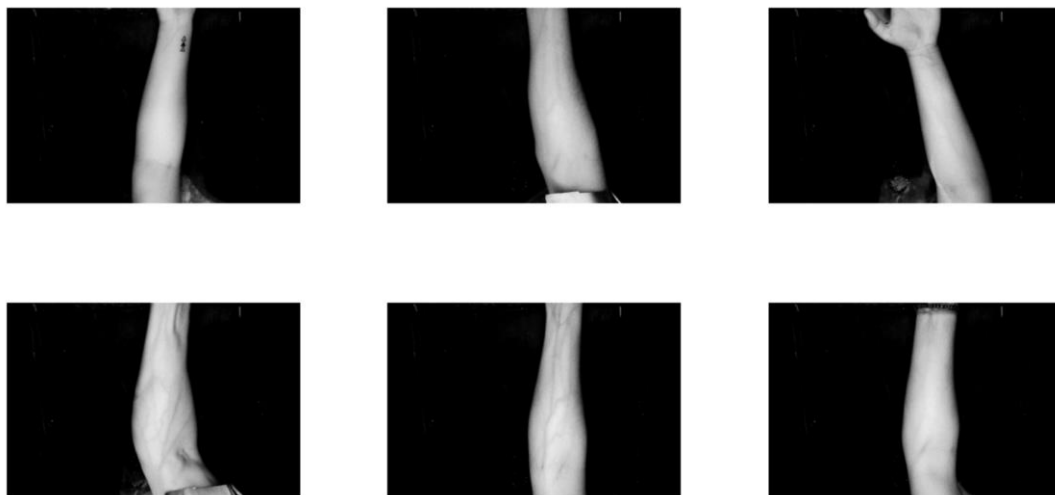


Fig. 2 Examples of images from the Forearm Veins dataset.



Fig. 3 Examples of vein masks from the Forearm Veins dataset.

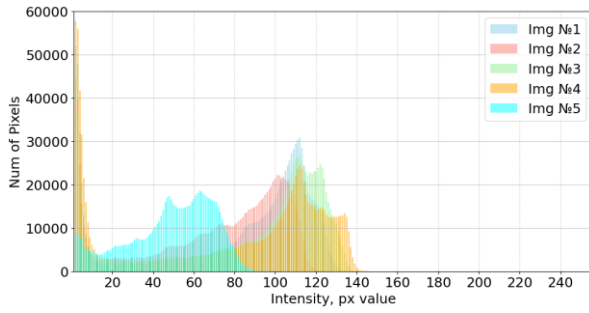


Fig. 4 Comparison of histograms of 5 images from the Forearm Veins dataset.

Fig. 5 presents the radial profiles of the spatial spectra for five images from the dataset. The radial profile is a one-dimensional slice of the two-dimensional FFT spectrum, taken along the radius at a 45° angle. This method simplifies the analysis by reducing the two-dimensional spectral data to a one-dimensional plot, avoiding the need for visual interpretation of intensities. The resulting graph shows the energy distribution across spatial frequencies: low frequencies represent large structures (e.g., background), while high frequencies correspond to fine details (e.g., vein edges).

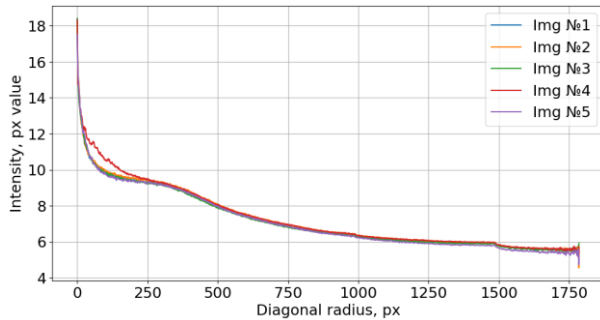


Fig. 5 Comparison of radial profiles of FFT spectra of 5 images from the Forearm Veins dataset.

The figures reveal significant differences in image histograms, with black tones dominating in all cases. However, the radial spectra profiles exhibit external similarities. An adaptive approach is suggested for histogram processing. Additionally, the similarity of spatial spectra suggests potential for applying FFT-based filters. Efficient FFT algorithms make real-time image processing feasible.

In our algorithm, a spatial high-frequency Gaussian filter is first applied to the original image. The FFT image of this filter can be calculated as follows [25]:

$$H(u, v) = 1 - \exp\left(\frac{-D^2(u, v)}{2D_0^2}\right). \quad (1)$$

Following the application of the Gaussian high-pass filter (HPF), the resulting image is processed using the Adaptive Contrast Enhancement (ACE) algorithm. ACE is an adaptive image processing technique that separates the image into low-frequency and high-frequency components. The low-frequency component is

determined as the local mean brightness of pixels within an $n \times n$ rectangular neighborhood (with n being odd), centered on the current pixel $x(i, j)$ [26]:

$$m_x(i, j) = \frac{1}{((2n+1)^2)} \sum_{k=i-n}^{i+n} \sum_{l=j-n}^{j+n} x(k, l). \quad (2)$$

The high-frequency component is calculated as the difference between the original pixel brightness value $x(i, j)$ and its local mean $m_x(i, j)$. To enhance contrast, a gain factor $G(i, j)$ is applied, which can be either constant or dependent on the local standard deviation (LSD) [26]:

$$f(i, j) = m_x(i, j) + G(i, j) \times (x(i, j) - m_x(i, j)). \quad (3)$$

A constant $G(i, j)$ may lead to ringing artifacts in the image. Consequently, the LSD is generally utilized in computations, as shown below [26]:

$$f(i, j) = m_x(i, j) + \frac{D}{\sigma_x} (i, j) \times (x(i, j) - m_x(i, j)). \quad (4)$$

where D is constant value, and LSD σ_x [26]:

$$\sigma_x^2(i, j) = \frac{1}{((2n+1)^2)} \sum_{k=i-n}^{i+n} \sum_{l=j-n}^{j+n} (x(k, l) - m_x(i, j))^2. \quad (5)$$

The Wallis filter [27] is subsequently applied to the processed image to enhance local contrast. The Wallis filter is an adaptive method of local contrast image processing that adjusts brightness and contrast based on the statistical characteristics of each local region. Unlike global methods, which apply uniform parameters to the entire image, the Wallis filter accounts for local mean values and standard deviations. This allows it to preserve details in both low-contrast and high-contrast areas.

The filter may be implemented as follows [27]. The original image is divided into square blocks of size $n \times n$, for which local statistics are calculated - namely, the local mean $m_{original}$ and the standard deviation $s_{original}$. For each pixel in the central block, new brightness values are computed using target parameters: the desired mean m_{target} and the desired standard deviation s_{target} .

The general expression for the transformation can be written as follows [27]:

$$i_{wallis}(x, y) = i_{original}(x, y) \times r_1 + r_0. \quad (6)$$

where the coefficients r_1 and r_0 are determined by a pair of Eqs. [27]:

$$r_1 = \frac{(c \times s_{original})}{(c \times s_{original} + \frac{s_{target}}{c})}, \quad (7)$$

$$r_0 = b \times m_{target} + (1 - b - r_1) \times m_{original}.$$

The parameter c controls the degree of contrast: small values ($c \rightarrow 0$) reduce contrast, making the image appear grayer, while values close to 1 enhance contrast. The parameter b determines the balance between target values and the original data: when $b = 1$, the output image fully matches the target parameters, whereas when $b = 0$, the original image is preserved. This filter has not been applied to vein visualization in previous researches.

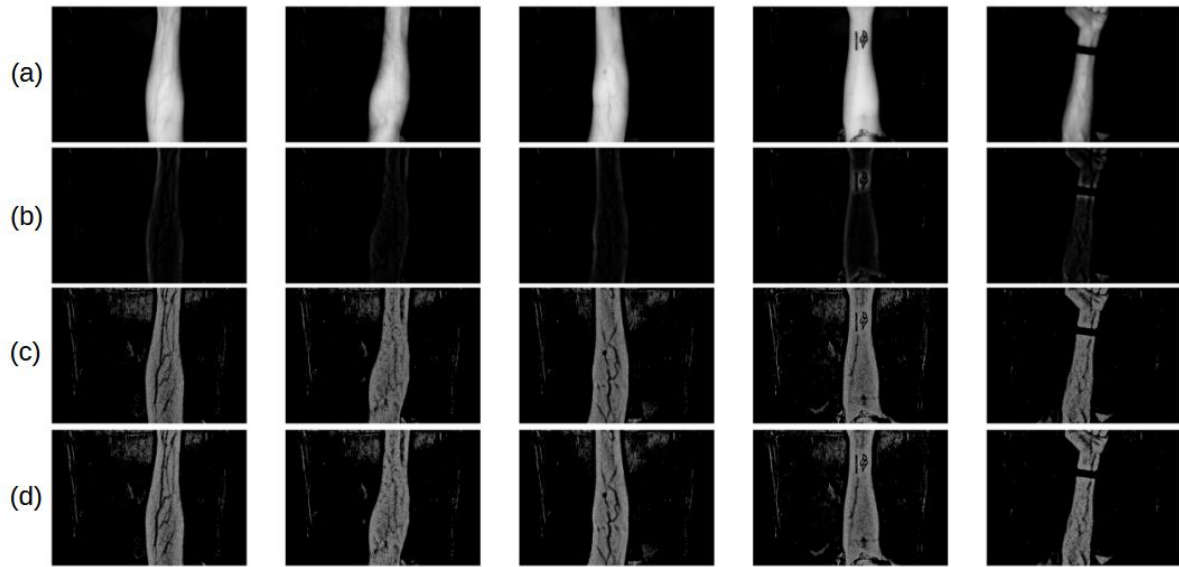


Fig. 6 A step-by-step illustration of the algorithm's operation: (a) raw image, (b) Gaussian HPF, (c) ACE, (d) Wallis filter.

However, it has proven effective in edge detection for brick walls and stained glass, tasks that are similar to vein visualization [27].

A step-by-step illustration of the algorithm's operation for five images from the original dataset is shown in Fig. 6.

The proposed algorithm effectively enhances vein contrast relative to the skin surface, as confirmed by visual analysis of the processed images: veins are clearly distinguishable in all dataset images. To the best of our knowledge, this is a novel method that not been reported in the literature previously. Furthermore, the use of optimized FFT and convolution operations from the OpenCV library ensures computational efficiency, making the algorithm suitable for deployment on low-resource devices.

2.3 Evaluation

To validate the image processing algorithms, an evaluation method was developed. The main idea behind the method is to compare the 8-bit processed images with binary reference images. The dataset includes vein mask annotations corresponding to 70 NIR digital photographs of 100. Reference images for evaluation were generated using the following procedure.

The dataset images were first processed to extract hand masks devoid of veins and background artifacts. This was achieved through automated processing using windowed rank percentile filtering. The filter aperture size was set to 65×65 , enabling the smoothing of minor background artifacts.

The percentile t for filtering an image $i(x,y)$ is determined as follows:

$$t = 2 \times \frac{\sigma(x,y)}{\max(x,y)}. \quad (8)$$

The general concept of the percentile filter is as follows. A sliding window moves across the image. For

each window, a new value is calculated for the pixel, which corresponds to a specified percentile of the sorted pixel values within the window. For example, a percentile filter with a specified percentile of 0.5 is equivalent to a median filter.

Global threshold binarization was applied to the image, with the threshold determined as the standard deviation of the image intensity, $\sigma(x,y)$. This approach enabled the extraction of forearm masks.

A forearm mask is a binary image in which pixels corresponding to the forearm region – including veins and surrounding tissues – are assigned the maximum intensity value in grayscale representation, while all other pixels are set to zero.

Vein masks, available for 70 images in the original dataset, were subtracted from the forearm masks. This operation produced reference images in which regions corresponding to veins were set to 0. The remaining pixels represented either background or superficial tissues. The resulting reference images are presented in Fig. 7.

Two types of errors were calculated: the error in forearm processing and the error in vein processing. The root mean square error (RMSE) was evaluated for each type:

$$RMSE = \sqrt{\frac{1}{M \times N} \sum_{i=1}^M \sum_{j=1}^N (i(i,j) - r(i,j))^2}. \quad (9)$$

Here, M denotes the image height, and N represents the image width. The terms $i(i,j)$ and $r(i,j)$ refer to the image and the reference image, respectively.

To evaluate the error associated with the forearm region, a specific area corresponding to the forearm mask is selected, excluding the vein mask region. The reference is defined as a rectangular array where all elements are equal to 255. This setup allows for assessing how closely the pixel values corresponding to the skin in the image approach the brightness value of 255.

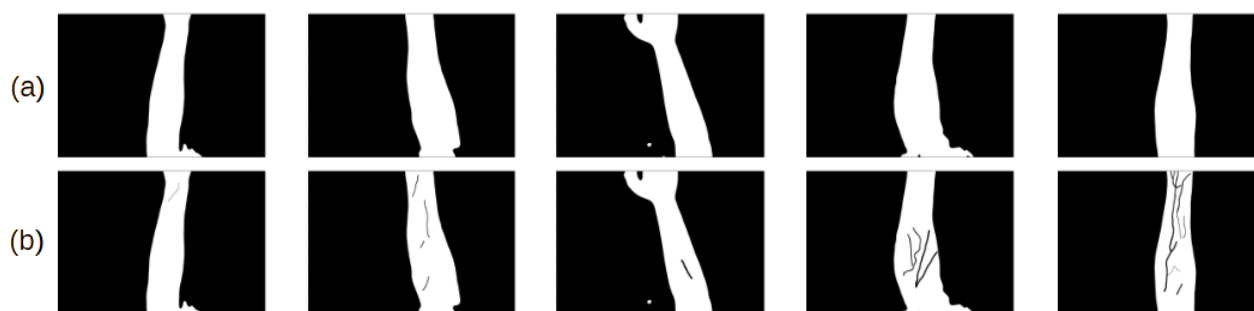


Fig. 7 (a) Forearm masks, (b) ideal reference images.

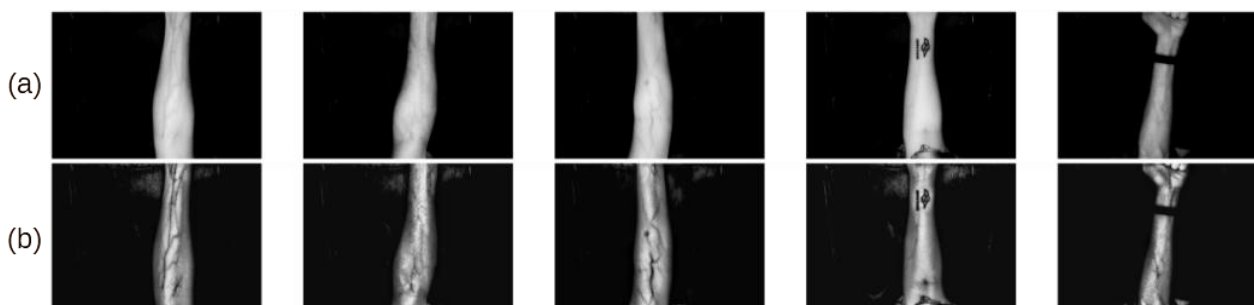


Fig. 8 Image processing with CLAHE: (a) before and (b) after.

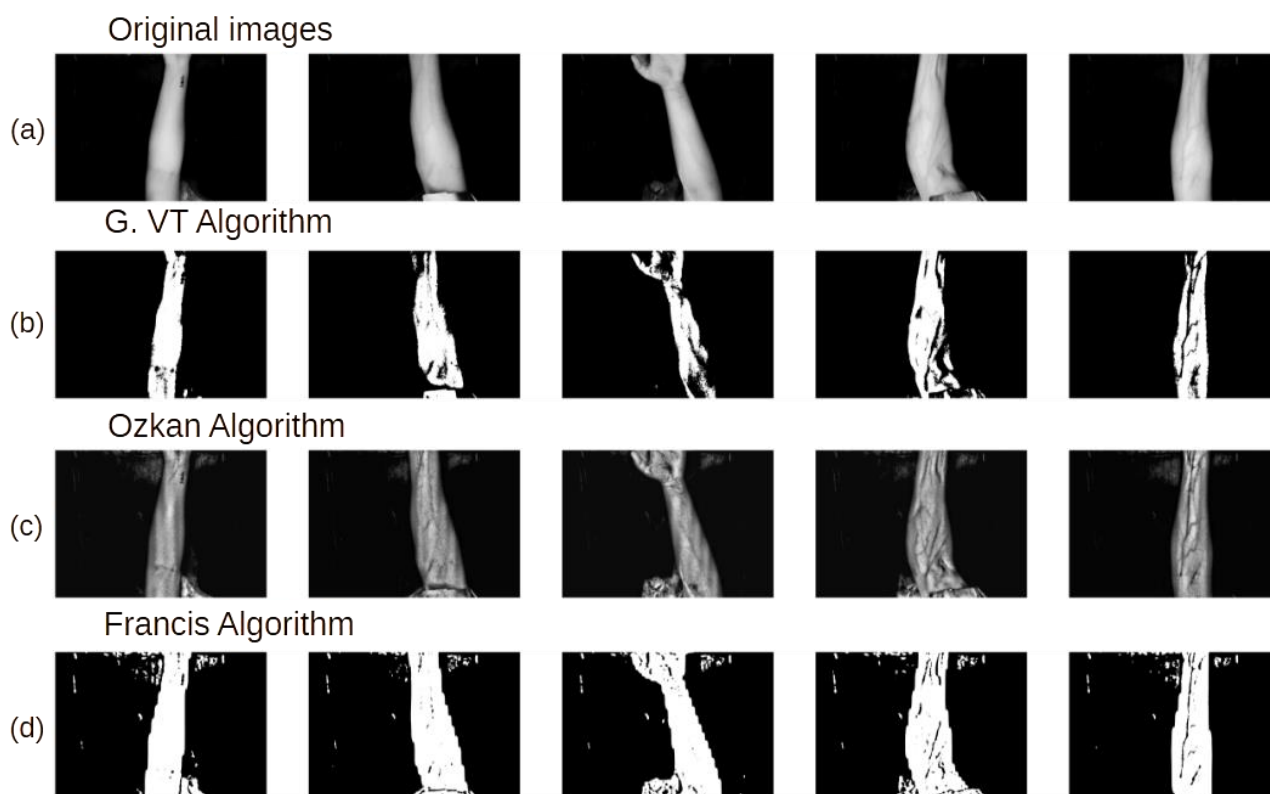


Fig. 9 (a) Original images vs (b) VT, (c) Ozkan, (d) Francis algorithms.

For evaluating the error associated with veins, the region corresponding to the veins is isolated. In this case, the reference is a rectangular array consisting entirely of zeros. This enables the assessment of how closely the pixel values corresponding to blood vessels in the image approach the brightness value of 0.

The RMSE value thus represents the root mean square deviation of the pixel values from the desired, reference ones.

To compare the efficiency of different algorithms, image processing was performed using the CLAHE algorithm was implemented separately. In general,

CLAHE divides the input image into equal rectangular regions, referred as tiles. Histogram equalization is then performed for each tile. During histogram equalization, a contrast enhancement threshold is used. The main idea behind thresholding is that the number of pixels at each brightness level in the current tile's histogram should not exceed a certain predetermined value. This approach prevents excessive histogram amplification in tiles with nearly constant brightness levels. The OpenCV implementation of the algorithm also includes bilinear interpolation of the boundaries between adjacent tiles. An example of image processing using the CLAHE algorithm for five images is shown in Fig. 8.

In addition, to enable a comprehensive comparison of algorithm performance on the ForearmVeins dataset, the VT, Francis, and Ozkan algorithms [4, 9, 12] were implemented in a simplified format, excluding ROI detection methods. These algorithms were chosen due to the fact that a complete description of the processing steps is available for them. Examples of the results obtained using these algorithms are shown in the Fig. 9. The performance of these algorithms is also evaluated.

This evaluation approach was designed to provide an objective assessment of the quality of visualization. It takes into account not only the determination of vein

edges but also the influence of the background. Skin and superficial tissues can significantly affect visualization. Unlike most existing studies, where result evaluation is either entirely absent or limited to analyzing vessel segmentation accuracy, the proposed approach is more versatile. It allows for simultaneous assessment of how well detected veins correspond to reference data. It also evaluates the correctness of background processing. This is particularly important because the intensity distributions of forearm areas in the original images overlap with those of vein regions. Such overlap directly impacts the quality of visualization and image processing requirements. The developed algorithm addresses this issue. It allows to identify a clear boundary on the histogram between regions corresponding to veins and those belonging to surrounding biological tissues.

3 Results and Discussion

The following results were obtained during the experiment. The developed algorithm achieves a significant reduction in pixel brightness error for veins. However, it slightly increases the pixel brightness error for skin areas. These effects are illustrated in Figs. 10 and 11.

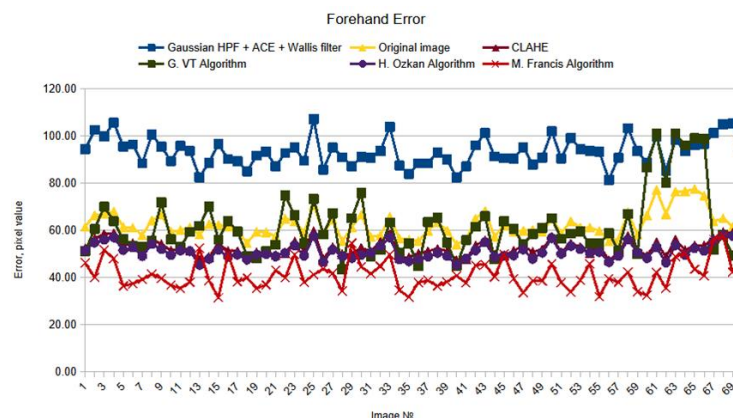


Fig. 10 Comparison of skin brightness error for five cases: without preprocessing, with preprocessing using the developed algorithm (Gaussian HPF + ACE + Wallis filter), with CLAHE, and with the methods proposed by Francis, Ozkan, and VT.

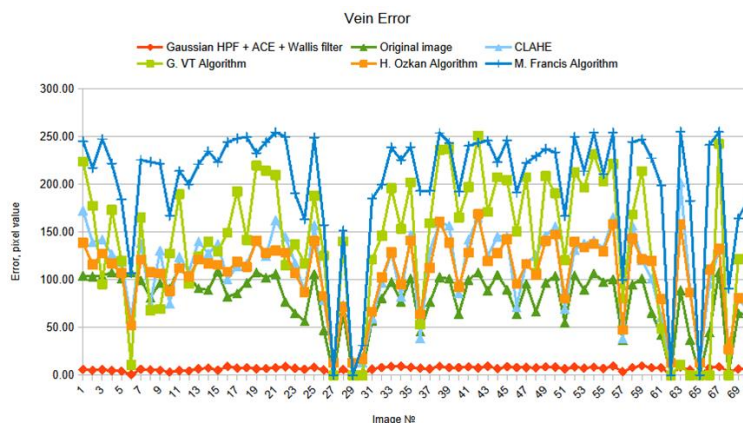


Fig. 11 Comparison of vein darkness error for five cases: without preprocessing, with preprocessing using the developed algorithm (Gaussian HPF + ACE + Wallis filter), with CLAHE, and with the methods proposed by Francis, Ozkan, and VT.

Pixel brightness error for skin areas is defined as the RMSE (Eq. (9)) calculated for pixels belonging to the skin area on the forearm, excluding regions corresponding to veins and background. The reference brightness level is set to 255, which represents the maximum possible value for an 8-bit image.

Pixel brightness error for veins is defined as the RMSE (Eq. (9)) calculated for pixels belonging to the vein area on the forearm, excluding regions corresponding to skin and background. The reference brightness level is set to 0, which represents the minimum possible value for an 8-bit image.

These definitions imply that the RMSE metric serves as a measure of how closely the pixel values align with the target reference levels for 8-bit image. Specifically, the RMSE value thus represents the root mean square deviation of the pixel values from the desired, reference ones.

All results are presented graphically to ensure clarity. In these graphs:

- The x-axis corresponds to the image number from the ForearmVeins dataset.
- The y-axis represents the error estimate.

This graphical representation offers a clearer and more intuitive interpretation than tabular data representation.

The results from the figures demonstrate the following:

- The vein darkness error for all images, after preprocessing using the developed algorithm, is close to 0.
- The skin brightness error increased from an average of 62 to an average of 93 (with preprocessing).

Key considerations regarding pixel values are as follows. Vein pixel brightness should ideally be close to 0, while skin pixel brightness should ideally approach 255. However, even if skin pixel brightness values are significantly lower than 255, human perception of the images is unlikely to be impaired. Taking this into account, the developed algorithm significantly enhances vein visualization efficiency. Furthermore, the processed images show more uniform pixel distribution compared to both the original images and those processed using CLAHE.

After processing the images using the developed algorithm, the forearm veins images' histograms converged to a more uniform shape. The histograms of all images were found to be right-skewed, with a uniform range of intensity values. A significantly larger number of pixels now have a value of zero. This makes it easier to differentiate veins from skin, as most vein pixels now correspond to a value of 0. In the original images, vein brightness varied widely, complicating the differentiation between veins and skin. The uniformity of the histograms allows for simple calculations to adjust the overall image brightness.

Visual analysis and RMSE evaluation reveal the following. The developed algorithm effectively separates pixels corresponding to veins from those representing the forearm. The deviation of vein-associated pixels from zero is minimal, indicating stable vein detection, as

confirmed by the graphs (Figs. 8, 10). By normalizing vein-associated pixels to a single level, the algorithm solves the problem of vein detection and simplifies subsequent automatic segmentation, if required.

Algorithms including binarization methods (VT, Francis) demonstrate generally unstable results. As shown in Fig. 8, vein error values can vary significantly, ranging from 75 pixel values (excluding images with very few vein-associated regions) to 250 for both the VT and Francis algorithms. In contrast, the error for the forearm region remains stable for both algorithms, as shown in Fig. 7. Due to the manual threshold selection, visualization quality varies significantly across different images. Additionally, the presence of a dark background adversely affects the performance of the Francis algorithm. While the article reports satisfactory visualization quality, quantitative metrics are not particularly informative in this case. This is because the resulting 8-bit images have only two brightness levels: 0 and 255 pixel values. Quantitative evaluation primarily highlights the high likelihood of incorrect pixel classification during binarization, as evidenced by the large errors observed in the graphs. In this case, for the given dataset, the obtained results indicate low reliability of the visualization outcomes, with a significant number of incorrectly classified pixels during binarization. This implies that the automation of image processing using these methods is challenging.

The application of CLAHE, as well as the Ozkan algorithm enhanced with bilateral and median filtering, visually improves image quality. However, these methods fail to provide reliable separation of pixel groups, as shown in Figs. 7, 8. Specifically, vein brightness does not approach zero, indicating no significant improvement in image contrast. The application of CLAHE does not ensure that veins will appear perfectly black if their original brightness overlaps with the background. Consequently, no effective image segmentation occurs.

In the original images, the pixel levels corresponding to veins overlap with those of the surrounding biotissues. Moreover, the brightness range of the vein pixels on the histogram may be in an unpredictable range. A similar situation was observed when processing with the Ozkan algorithm. However, for the developed algorithm, the majority of vein-associated pixel levels are reduced to zero, while the mode of the pixel level for surrounding biotissues is located around 9–10.

On the other hand, the developed algorithm, based on the Gaussian high-pass filter, slightly increases the error for skin areas but significantly reduces the error for veins, in comparison with original image. This results in higher-contrast images, enabling clear differentiation between the background and veins.

Furthermore, developed algorithm can be efficiently implemented using fast convolution techniques and FFT. It shows strong potential for real-time applications.

The developed image processing algorithm for vein visualization potentially demonstrates high efficiency in specialized medical applications, such as supporting

intravenous injections and preoperative planning for phlebectomy. Notably, the algorithm consistently achieves excellent results on the current dataset, correctly processing all provided forearm images. However, it is important to consider that this method has been tested only on a limited sample, primarily using data obtained from patient forearms. This imposes certain limitations on its applicability to other body parts, where anatomical features and imaging conditions may differ significantly.

In real clinical settings, the algorithm may face additional challenges not accounted for in this study, such as variations in lighting, skin types, or patient movements. To address these limitations, two main directions for further development are proposed: first, expanding the dataset size to cover a broader range of clinical cases, and second, integrating the algorithm into actual medical devices for long-term practical testing. This approach will not only verify the system's reliability under real-world conditions but also enable the collection of valuable data for subsequent algorithm improvements.

4 Conclusion

This work presents an efficient digital image processing algorithm designed to improve the visualization quality of subcutaneous veins in the near-infrared spectrum. The proposed method significantly enhances the contrast between veins and surrounding tissues, which is essential for accurate and reliable visualization.

To evaluate the algorithm's performance, a quantitative assessment method was proposed. The developed algorithm reduces vein brightness error by 20–40% compared to the original images. After processing, the brightness of vein-related pixels approaches 0, significantly simplifying their differentiation from skin areas. This is confirmed both by numerical metrics and visual analysis of the images.

The error for skin areas increases by 10–15%. However, this change remains acceptable because it does not impair human perception of the image. Moreover, the histograms of the processed images converge to a uniform shape, enabling simple brightness transformations to be applied to the image.

Comparison with the CLAHE algorithm, widely used in vein visualization studies, highlights the superiority of the developed algorithm. While CLAHE increases vein error by 10–40% and reduces skin error only marginally (5–10%), the proposed algorithm achieves better differentiation between veins and the background. This improvement is made possible by applying Gaussian high-pass filtering, which effectively enhances fine details.

A comparative analysis with other algorithms has been performed. The developed algorithm demonstrates superior performance in terms of robustness, adaptability, and efficiency of pixel separation. Furthermore, the developed algorithm effectively reduces the brightness of most pixels related to veins to zero. This allows for reliable segmentation of pixels related to veins from pixels related to surrounding biological tissues. This differentiates developed algorithm from most other existing algorithms in a beneficial way.

The algorithm is expected to perform well due to the use of fast convolution techniques and the Fast Fourier Transform, which are implemented in OpenCV. This makes it potentially suitable for real-time image processing, which is a critical requirement in clinical settings such as venipuncture and blood sampling procedures.

Disclosures

All authors declare that there is no conflict of interest in this paper.

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