Optical clearing of biological tissues: prospects of application in medical diagnostics and phototherapy

E.A. Genina^{1,2*}, A.N. Bashkatov^{1,2}, Yu.P. Sinichkin¹, I.Yu. Yanina³, V.V. Tuchin^{1,2,4}

¹ N.G. Chernyshevsky Saratov State University, 83 Astrakhanskaya Str., Saratov, 410012, Russia

² Tomsk State University, 36 Prosp. Lenina, Tomsk, 634050, Russia

³ V.I. Razumovsky Saratov State Medical University, 112 Bolshaya Kazachya Str., Saratov, 410012, Russia

⁴ Institute of Precision Mechanics and Control, Russian Academy of Sciences, 24 Rabochaya Str., Saratov, 410028, Russia

* e-mail: eagenina@yandex.ru

Abstract. A review of specific features and methods of optical clearing and related interaction of light with tissues is presented. Physical and molecular mechanisms of immersion, compression, and photodynamic/photothermal optical clearing of some fibrous and cellular tissues are discussed. The possibility of efficient control of the tissue optical properties, particularly, the reduction of light scattering in tissues is demonstrated, which facilitates the increased efficiency of various optical visualisation methods (optical biopsy) used in medical purposes. © 2015 Samara State Aerospace University (SSAU).

Keywords: tissue, optical clearing, optical diagnostics, imaging.

Paper #1992 received 2014.12.26; revised manuscript received 2015.02.03; accepted for publication 2015.02.05; published online 2015.03.28.

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Contents

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1 Introduction: fundamentals of optical clearing of tissues and cells

During the last 25 years the interest to the development and application of optical methods in clinical functional imaging of physiological conditions, diagnostics and therapy of cancer, and other diseases is permanently growing [1-3]. It is caused by the unique informativity, relative simplicity, safety, and sufficiently low cost of optical instruments, as compared, e.g., to X-ray computer tomography or magnetic resonance tomography (MRT). However, the main limitation of optical diagnostic methods, including the optical diffusion tomography, optical coherence tomography (OCT), confocal microscopy, reflection spectroscopy, etc., is the strong scattering of light in biological tissues and blood that reduces the contrast, spatial resolution, and probing depth [3-5].

The scattering coefficient (μ_s) and the scattering anisotropy factor (g) mainly depend upon the refractive index mismatch between the components of the cells that form the tissue, such as the plasma membrane of the cell, mitochondria, nucleus, other organelles, cytoplasm, and extracellular fluid. In fibrous tissues (stroma of eye sclera and cornea, dermis, *dura mater*, connective tissue of vascular walls, fibrous components of muscle tissue and mammary gland, cartilage, tendon, etc.) the scattering is caused by the refractive index difference between the interstitial fluid or cytoplasm and the extensive chains of scleroproteins (collagen,

elastin, and reticulin fibrils) [3, 6]. The refractive index values for nuclei and cytoplasm organelles of animal cells, containing nearly the same amount of proteins and nucleic acids, lie within the relatively narrow interval from 1.38 to 1.41 [7]. In particular, for the nucleus the refractive index is $n_{nc} = 1.39$ [8], and for the cytoplasm $n_0 = 1.35-1.37$ [3]. The scattering particles (organelles, protein fibrils, membranes, and globules) have greater density of proteins and lipids and, hence, higher refractive index ($n_s = 1.39-1.47$) in comparison with the base substance of the cytoplasm [6]. The refractive index values of the connective tissue fibrils lie in the range 1.41-1.53 and depend upon the degree of hydration of their major component, the collagen [9]. The refractive index of the interstitial fluid and the blood plasma amounts to nearly 1.33-1.35 depending on the wavelength [3, 10]. The main scatterers in the blood are the red blood cells (erythrocytes), which are acaryocytes, containing 70% of water, 25% of haemoglobin, and 5% of lipids, sugars, salts, enzymes, and proteins [11]. The refractive index values of dehydrated erythrocytes at the wavelength of 550 nm fall within the range 1.61-1.66 [12]. The refractive index of the haemoglobin solution with the concentration of 32 g/dL, which is a typical concentration of haemoglobin in the erythrocyte, amounts to nearly 1.42 [13]. For the human blood the refractive index is 1.36-1.40 depending on the wavelength [3].

Numerous methods have been developed to increase the tissue probing depth [14]. For example, during a long time the limit probing depth in multiphoton microscopy did not exceed 100 μm [15], however, the combination of multiphoton excitation of fluorescence with very high efficiency of light collection increased the imaging depth in a scattering medium to 2 mm [16, 17]. The OCT allows the study of internal structure of tissues to the depth up to 3 mm with the spatial resolution 5-20 μm without disturbing the tissue integrity [18]. The probing depth up to a few centimetres is provided by the multimodal imaging method, combining the light absorption with acoustic detection (referred to as photoacoustic tomography) [19].

One of simple and efficient methods of solving the problem of increasing the depth and quality of intratissular structure imaging, as well as of increasing

the precision of spectroscopic information from the deep tissue layers and the blood, is the temporary reduction of the tissue light scattering [20-22]. In nonlinear spectroscopy and imaging, as well as in high-precision laser surgery (particularly, in cell nanosurgery), the reduction of scattering in the tissue facilitates the decrease of the strongly focused laser beam divergence and provides its precise focusing onto the object, which essentially improves the efficiency of the procedure and allows the reduction of the radiation energy, necessary for successful visualisation or photo-induced effect [21-27].

According to Web of Science, PubMed and other sources, the interest to the optical clearing methods is permanently growing, which is caused by the progress of optical and laser technologies for application in biology and medicine (see Fig. 1).

Fig. 1 Approximate evaluation of the number of publications related to the optical clearing of tissues from 1955 to August 2014. The dependences are plotted using the databases Web of Science, PubMed, and other available sources.

Numerous examples of physical and chemical impacts that allow one to control the scattering properties of tissues are reported. They include compression [28, 29], stretching [30], dehydration [31, 32], coagulation [33], immersion with biocompatible chemical agents [20-27, 34-36], as well as photochemical [37, 38] and photothermal [38] clearing.

The majority of methods reducing the tissue scattering are based on the matching of the refractive indices of the tissue components, either due to replacing the interstitial fluid with the immersion agent, possessing a higher refractive index, or due to increasing the concentration of proteins and mucopolysaccharides in the interstitial fluid as a result of water diffusion from the tissue caused by the osmose, making the refractive index of the interstitial fluid closer to that of the fibrils. In addition, the optical homogeneity can increase due to the compaction of scattering centres (e.g., collagen fibres) due to both pressing out the interstitial fluid from the affected volume and the dehydration at the expense of the agent

impact or water evaporation. [22, 25, 26, 28-32, 39]. The particular mechanisms of optical clearing depend on the tissue type and the method used.

In the present review we discuss the physical and molecular mechanisms of immersion, compression, and photodynamic/photothermal methods of optical clearing of some fibrous and cellular tissues, as well as the application of these methods for increasing the probing depth and resolution power of optical diagnostic methods, such as optical coherence, projection, and photoacoustic tomography, fluorescence imaging, nonlinear and Raman microscopy, and terahertz imaging. This review is different from earlier published and cited ones (for example, Refs. [36, 39]) extended bibliography due to addition of new references and sections. In particular, the mechanisms and features of compression and photodynamic/photothermal clearing are described in details; sections on methods of OCA diffusion coefficient evaluation and applications of optical clearing technique (THz spectroscopy, projection and photoacoustic tomography etc.) are extended significantly.

2 Immersion clearing

Following the development of immersion refractometry applied to cells, in 1955 Barer et al. [40] have first proposed the optical clearing of cell suspension by means of the protein solution having the same refractive index as the cell cytoplasm. In early 1990s the method of immersion optical clearing was first applied to the eye sclera and cornea [41].

After the appearance of the tissue optical clearing idea many research teams joined the intense studies of the specific features and mechanisms of this phenomenon and demonstrated the capabilities of the method in increasing the probing depth or contrasting the image of optical inhomogeneities inside a scattering medium [39, 42-88].

New original results obtained using the combination of optical clearing with the known optical visualisation methods, such as laser speckle-contrast imaging [34, 71, 75, 77, 79], OCT [20, 29, 35, 59-61, 65, 69, 80, 82], microscopic imaging [23, 24, 83, 84], ultra-microscopy [85-87], etc., demonstrated high potentiality of their mutual use not only for getting high-resolution structure and functional tissue images *in vitro* [72, 73, 83-87], but also for optical imaging and diagnostics *in vivo* [25, 34, 45, 50, 59, 61, 65, 68, 70, 71, 75-77, 79-81]. Table 1 shows the increase of the light penetration depth, caused by clearing agents in some diagnostic methods.

The studies of tissue scattering kinetics under the penetration of immersion agents allowed the development of techniques for assessing the diffusion coefficients and permeability [35, 43, 47, 88-94]. Using these techniques, the diffusion rates of glucose and other medicinal preparations and immersion fluids were determined for the tissues of eye [91-96], skin [45, 97, 98], *dura mater* [88], and other tissues [99, 100]. The monitoring of optical clearing agent diffusion with high resolution in time and depth allows differentiation

between healthy and pathologically modified biotissues [101-105].

The optical immersion clearing, as already mentioned above, is based on the impregnation (immersion) of the tissue with a biocompatible chemical agent, possessing sufficiently high refractive index to match the refractive indices of the scatterers and the surrounding medium, penetrating into the interstitial liquid of the tissue. Commonly, the optical clearing agent (OCA) has hyperosmotic properties [21, 22]. Many chemical substances used in cosmetology in the composition of different preparations satisfy these conditions. These agents can be roughly divided into polyatomic alcohols (glycerol, polyethylene glycol (PEG), polypropylene glycol, combined mixtures on the base of polypropylene glycols and polyethylene glycols, mannitol, sorbitol, xylitol) [56, 57, 76, 84, 88, 103, 105-108], sugars (glucose, dextrose, fructose, ribose, saccharose) [82, 88-91, 94, 96, 97, 99, 100, 104, 109-111], organic acids (oleic and linoleic acid) [105, 113, 114], other organic solvents (dimethyl sulphoxide (DMSO)) [105, 114-117], and X-ray contrast agents (verografin, trasograph) [6, 42-44].

At present several physical and chemical mechanisms of the light scattering reduction under the action of OCA are proposed and thoroughly described [29, 31, 32, 39, 43, 46, 47, 55, 56, 58, 63, 67, 72, 76, 78, 84, 88, 106-112, 118-122]: dehydration of tissue components, partial replacement of interstitial fluid with the immersion agent, and structure modification or dissociation of collagen. The first mechanism is related to the hyperosmotic properties of the OCA [31, 36, 39, 56, 120]. The contact of hyperosmotic OCA with the tissue surface causes the water diffusion from the tissue. These processes produce fast and considerable clearing effect, since, first, the concentration of salts and proteins dissolved in the interstitial fluid increases and, therefore, the refractive index of the interstitial fluid becomes closer to that of the scattering fibrils, and, second, the weight and the thickness of the tissue decrease, the tissue becomes denser, and the ordering of scattering components increases [36, 39].

Rylander et al. [31] compared the optical transmission of the rat skin under the application of DMSO and glycerol with that under dehydration. It was shown that in the course of natural water evaporation the light transmission through the skin sample increased in the same way as under dehydration caused by the OCA effect, but during a longer time.

In order to analyse the water loss in the process of tissue optical clearing quantitatively, the authors of Ref. [67] used the optical spectroscopy in the near IR range. Measuring the skin reflection coefficient at the wavelengths $\lambda_1 = 1100$ and $\lambda_2 = 1936$ nm (the water absorption band), Xu and Wang used the double-wave analysis to assess the water content in the skin. The authors demonstrated that the change of the water content in the skin correlated with the optical clearing effect during the first minutes, namely, under the impact of 80% solutions of glycerol and propylene glycol the

water content at first rapidly decreases, and then the dehydration process becomes slower.

The kinetics of skin dehydration degree under the action of 88% aqueous solution of glycerol and air, studied by Genina et al. [56] basing on the weight measurements of human skin samples (see Fig. 2a), allowed the assessment of the characteristic time τ of the process, i.e., the time, during which the dehydration degree increases by e times. Under the action of OCA τ amounts to 193.4 ± 12.3 hours, while under the conditions of natural evaporation of interstitial water this parameter equals 1408.9 ± 36.3 hours.

Fig. 2 Dehydration kinetics of human skin samples with intact (a) and perforated (b) epidermis under the action of 88% glycerol solution (1) and in the course of interstitial water evaporation in air (2). The symbols correspond to the averaged experimental data; the curves show the result of approximation. The vertical lines present the standard deviation (from the data of Ref. [56]).

At the same time, it is seen from the Fig. 2 that, in contrast to the natural drying in air, under the action of the OCA no full dehydration occurs, since a part of the OCA penetrates into the interstitial space and is mixed with the interstitial fluid. It is known, that most of the OCAs are hygroscopic and in the humid environment they adsorb water molecules till the saturation is achieved. Thus, for glycerol the saturation level

amounts to 55 and for propylene glycol 32 percent in volume [123]. Therefore, OCAs inhibit complete dehydration by keeping a part of water in the tissue.

For fibrous tissues, such as sclera, *dura mater*, dermis, etc., both processes, namely, the water loss and the diffusion of the hyperosmotic agent into the skin, occur simultaneously, but the mechanism of the interstitial fluid replacement with the OCA solution is prevailing for all the agents used, since their molecular size is much smaller than the mean separation between the fibrils [3, 43].

In the case of transepidermal delivery of hydrophilic OCAs into the skin the diffusion is inhibited by the existence of the epidermis lipid barrier. The human skin dehydration at the delivery of the hyperosmotic OCA through the perforated *stratum corneum* (the perforation may be implemented, e.g., by fractional microablation) is presented in Fig. 2,b. The epidermis permeability improvement resulted in the accelerated diffusion of both water and 88% glycerol solution. Fig. 2 shows that when the samples with intact and perforated horny layer are dried under the identical conditions, their final dehydration degree should be practically similar. However, from the analysis of the dehydration characteristic time (for the perforated sample τ amounts to 71.2 ± 1.9 hours) it follows that the dehydration process in this case occurs by more than 20 folds faster than in the intact epidermis ($\tau = 1408.9 \pm 36.3$ hours). The value of τ for the sample with perforated epidermis under the action of the OCA was only 36.4 ± 0.7 hours, which is almost by 5.5 folds smaller than for the intact sample ($\tau = 193.4 \pm 12.3$ hours) [56].

In Fig. 2,b it is well seen that with the epidermis damaged the maximal value of dehydration degree under the evaporation of water from the tissue is essentially higher than under its osmotic removal. At the same time the skin dehydration rate due to evaporation is by nearly two times smaller than under osmotic water removal.

The study of water evaporation process allowed the separation of the dehydration mechanism of optical clearing from other mechanisms (i.e., the matching of refractive indices due to the penetration of OCA into the tissue and the collagen structure modification). The fact that attracts attention in the dehydration under the action of glycerol is that the degree of dehydration in the samples with perforated *stratum corneum* is smaller than that in the intact samples. As mention above, in both cases there are two counterpropagating fluxes, one of them is the water diffusion from lower layers of epidermis and from dermis into the surrounding solution, and the other one is the diffusion of the OCA from the surrounding solution into the tissue. Apparently, in the non-perforated sample the rate difference between these fluxes is greater than in the perforated one, since in the absence of the epidermis damage the drainage of water from the tissue considerably exceeds the glycerol delivery into the tissue. From the practical point of view, the topical injection of OCA into the skin through the channels in

the epidermis hampers the formation of strong concentration gradient between the interstitial fluid and the OCA solution, which reduces the tissue dehydration and can be considered as a positive factor for optical clearing of skin *in vivo*.

Mao et al. [107] focused their attention on the study of the efficiency of six polyatomic alcohols with hydroxyl groups (1-butanol, 1,4-butanediol, 1,3-propanediol, PEG-200, PEG-400, and glycerol) with molecular weights from 74 to 420 Da, and the refractive index values from 1.40 to 1.47, affecting the degree of skin optical clearing. For better *in vitro* modelling of the topical OCA application to the skin *in vivo*, the agents were applied to the pig skin epidermis, while the dermis was moistened with saline. The relative transmission of the samples, measured using the integrating sphere, was used to assess the efficiency of optical clearing [107]. The authors found that the glycerol, which is a triatomic alcohol, caused the maximal clearing effect, the monoatomic alcohol 1-butanol caused the smallest effect, and four diatomic alcohols caused intermediate clearing effect. After checking the interrelation between the optical clearing efficiency and the refractive indices or molecular weight of the OCAs under study, the authors concluded that the clearing effect, caused by alcohols, should be related to the number of hydroxyl groups rather than to the refractive index or the molecular weight value of the OCA.

In fibrous tissues the optical clearing process can also incorporate the variations in the molecular structure of the basic component of these tissues, the collagen fibres, including the reversible solvability of collagen in sugars and polyatomic alcohols. The OCA-induced destabilisation of collagen structure can lead to the additional reduction of optical scattering in a tissue due to the decrease of the basic scatterer size. [121].

The collagen fibres have complex self-organising structure and are the main scattering centres in tissues [112]. They are widely presented in different tissues, particularly, in fibrous ones, such as skin dermis and eye sclera. It is established that the hydrogen bond is the main bonding force between the triple collagen helices. The OCAs with multiple hydroxyl groups possess a greater negative charge that destabilises the highly-ordered collagen structure till its dissociation. Since the hydrogen bonds in the triple collagen helices belong to non-covalent interactions, the OCA-induced effect on the collagen dissociation can be easily reversed. Yeh et al. [109, 112, 121, 122] observed the dissociation of collagen fibres in the tissue *in vitro*, merged in glycerol. The subsequent replacement of glycerol with phosphate-buffer solution provided the restoration of the collagen structure.

Hirshburg et al. [110] performed mathematical modelling of molecular dynamics in order to clarify the formation of hydrogen bonds between the alcohol (glycerol, xylitol, and sorbitol) and collagen molecules. They divided the hydrogen bond bridges into different types depending on the position of hydroxyl groups, involved in the interaction, with respect to carbon

atoms. They associated the type number with the number of carbon atoms in the hydrogen bond bridge. It was found that the bridges with large number, built in between the collagen molecules in the helix, can break the collagen-collagen and collagen-hydroxyl bonds more efficiently than the bridges with smaller numbers. Thus, the alcohols having pairs of hydroxyl groups with longer carbon chain between them should be more efficient in optical clearing than the alcohols having adjacent hydroxyl groups. The results of the modelling explain the fact that 1,3-propanediol demonstrated the optical clearing potential twice as great as that of 1,2-propanediol, although both possessed similar molecular weight (76.10 Da), close values of the refractive index (1.44 and 1.43), and osmolality (8.3 and 8.7 Osm/kg). The reason is that 1,2-propanediol can form only the bridges of the type I, while 1,3-propanediol forms the bridges of the type II [110]. Since the modelling of molecular dynamics can clarify the specific features of interaction between the OCA and the structural elements of the tissue at the microscopic (molecular) level, it can be a powerful tool in the study of optical clearing mechanisms, particularly, for choosing new high-efficiency OCAs.

Of great practical interest is the optical clearing of hard tissues, such as bone, cartilage and tendon. The reduction of scattering in these tissues offers the possibility to develop minimally invasive methods of laser diagnostics and therapy of brain and other deeply located tissues. The possibility of studying brain blood circulation without damaging the skull bone was demonstrated by Wang et al. [81]. The experiments with laboratory mice *in vivo* have shown that the minimal microvascular diameter resolved in the observation through the bone tissue, preliminarily processed with the specially developed optical clearing solution, amounts to $14.4 \pm 0.8 \mu\text{m}$. The optical clearing of the human skull bone *in vitro* was studied by Genina et al. [57]. The authors have shown that the exposure of the ~ 5 mm thick skull bone sample to glycerol reduces the value of the transport scattering coefficients by 25% in the spectral range 1400–2000 nm. In the present case the main role in the clearing process is played by the replacement of water in the interstitial space with OCA due to the specific structure of the bone tissue that possesses higher porosity than soft tissues.

The transparency increase was studied in the samples of cartilage tissue of the human nasal septum and knee joint *in vitro* under the impact of trasograph [124] and in the rodent tail cartilage *ex vivo* under the impact of glycerol [112, 121]. The authors demonstrate the increase of the contrast in the image of the tissue by 2 – 2.5 folds during 1 minute for the transverse section and by 1.6 folds during 8 minutes for the longitudinal one [124] and the reversible disorganisation of collagen fibrils under the action of OCA [112, 121].

Optical clearing of the mouse tail tendon *in vitro* exposed to the glycerol solutions with different concentration was demonstrated in the papers by LaComb et al. [72], Nadiarnykh and Campagnola [73],

and Rylander et al. [31]. The authors managed to increase the probing depth in the second harmonic generation microscopy up to $450 \mu\text{m}$ at the expense of considerable reduction of the scattering coefficient (up to 130 folds at the wavelength 890 nm during 5 hours) and increasing the packaging density of fibrils (according to the data of electron microscopy, the volume fraction of fibrils increased from 0.65 to 0.9) [31].

Blood vessels and capillaries transpierce practically all tissues, and the blood is a strongly scattering medium, so that its optical clearing is a serious problem for successful application of optical technologies both in diagnostics and in therapy/surgery. The difference between the refractive indices of the erythrocyte cytoplasm and the blood plasma, as well as the specific size and structure of blood corpuscles explain the blood scattering properties [3, 10, 11]. The refractive index of the erythrocyte cytoplasm is determined mainly by the haemoglobin concentration [11, 13, 125]. The volume and the shape of an individual erythrocyte are determined by the osmolality of the blood plasma [125, 126]. The blood scattering is also dependent on the ability of the red blood cells to aggregate or disaggregate [127].

When an OCA is injected into blood, the refractive index of the blood plasma increases and becomes comparable with the refractive index of the red blood cells. For example, the total attenuation coefficient of whole blood, diluted by two folds with saline, under the addition of 6.5% glycerol solution decreased from 4.2 to 2.0 mm^{-1} , and, correspondingly, the optical probing depth at the wavelength 820 nm increased by 117%. For other OCAs tested (glucose, dextran, propylene glycol, and trasograph) the increase of the probing depth was from 20 to 150% [22, 128]. It was also shown that the introduction of chemical agents is able to change the size and aggregation properties of erythrocytes, which allows the control of the blood optical properties, too [128].

Bashkatov et al. [53] have shown that the minimal light scattering is observed at the concentration of glucose in blood equal to 0.65 g/ml. In this case the blood appears to be completely immersed, but due to the difference in spectral dependence of refractive indices of the glucose solution and erythrocytes the residual scattering is still present. Obviously, such high concentration of glucose can be applied only topically and during a short period of time to prevent the destruction of blood and vascular wall cells. Nevertheless, the use of endoscopic optical imaging systems (OCT or confocal microscopy) and the controlled injection of small glucose volumes into the vascular lumen near the region of imaging may essentially facilitate obtaining a high-contrast image of an atherosclerosis plaque through the layer of blood.

The same group of authors showed the possibility of using a small volume of haemoglobin as an OCA. This haemoglobin can be obtained as a result of local haemolysis of erythrocytes in a blood vessel near the

site of optical endoscopic probe [51]. The blood scattering coefficient reduction by 30-40% was demonstrated theoretically in the spectral region 400-1000 nm under the increase of the local haemolysis by up to 20% in the immediate proximity of the optical probe.

Impregnation of blood-saturated tissues, such as the liver, with solutions having different osmolality also leads to the matching of refractive indices and scattering coefficient reduction, but the effect is not as prolonged as in fibrous tissues [22].

The optical clearing of tissues *in vivo* is, generally, more complex, since in this case a significant role begins to be played by the additional factors, on the one hand, the physiological temperature that accelerates the diffusion of OCA, and, on the other hand, the metabolic reaction of the living tissue, washing the OCA out. These factors can essentially modify the kinetic characteristics and the magnitude of the clearing effect [25, 34, 45, 59, 61, 68, 70, 76, 77, 79, 80, 82, 97, 98, 108, 113, 118, 119]. In living tissues the refractive index is a function of the physiological or pathological condition of the tissue. Depending on the specific features of the tissue condition, the refractive index of scatterers and/or the base substance can change (increase or decrease), and correspondingly the light scattering will change [3, 22]. In addition, the introduction of some OCAs, in particular, glycerol and glucose, into a tissue affects the condition of microcirculation in the tissue, causing a transient stasis of the microvessels [50, 65, 71, 77, 108].

One of the most important problems in optical diagnostics and treatment is the reduction of skin scattering aimed at visualising the inhomogeneities hidden inside the skin or under it. However, it is rather difficult to obtain the optical clearing of skin using noninvasive or minimally-invasive means, since the *stratum corneum* of the skin epidermis is a natural barrier that impedes the OCA penetration into the dermis [129]. For *in vivo* applications of the optical clearing method one has to use direct exposure of the skin dermis, or intradermal OCA injection. However, at low concentrations OCAs do not provide sufficient optical clearing, while at high concentrations they can induce edema, chemical burn, partial necrosis and scarring [45, 65, 108]. To develop an efficient and safe way of breaking the integrity of the epidermis *stratum corneum* and accelerating the penetration of OCA into the dermis, various physical methods [56, 118, 130-140], chemical enhancers of permeability [114, 118, 141-147], and their combinations [118, 148, 149] have been proposed.

Chemical OCA diffusion enhancement is implemented using the agents that serve for increasing the tissue permeability in medicine and cosmetology [36, 39]. In particular, it is shown that Azone [141], oleic acid [142], DMSO [118, 143-146], ethanol [150], propylene glycol [147, 151], Thiazone [149] enhance the tissue permeability for OCAs and increase the efficiency of optical clearing.

The possible mechanism of diffusion enhancement using Azone may consist in increasing the fluidity of hydrophobic regions of the *stratum corneum* and the corresponding reduction of resistance to the penetration of agents [141]. For example, after 60 minutes exposure of *in vitro* skin to the mixture of 40% glycerol-Azone the absorption of light at the wavelength 1276 nm increased by 41.1%, and the diffuse reflection at the wavelength 1066 nm decreased by 29.3%, which considerably exceeded the values of these parameters under the application of more concentrated 80% aqueous solution of glycerol [141].

The oleic acid is a monounsaturated fatty acid that is widely used as a safe transdermal enhancer for drug delivery. In the region of skin clearing the synergetic effect of oleic acid as a promoter of OCA skin penetration was studied [142].

DMSO is a well-known agent widely used for improving the transdermal delivery of drugs. It is a polar aprotic solvent of *stratum corneum* lipids [143]. DMSO enhances the permeability of the *stratum corneum* for both hydrophilic and lipophilic agents [152, 153]. In addition, it possesses high refractive index and may serve as an OCA as well [144]. It also interacts with the highly organised structure of collagen fibres, changing the interfibrillar space at the sub-micrometre scale [145], which is of great importance for the skin optical clearing. However, the results on the DMSO optical clearing potential and safety are rather contradictory [106, 144, 154, 155]. In Ref. [106] it is shown that the optical clearing potential of DMSO is smaller than that of glycerol, propylene glycol, ethylene glycol, and some other OCAs. The application of high-concentration DMSO to the skin surface causes irritation, accompanied with epidermal spongiosis [154]. On the other hand, it was noticed that DMSO is a high-efficiency OCA of topical application, and no side effects under the 20-minute exposure of the rat skin surface *in vivo* were reported [144]. In spite of the controversial evaluation of the DMSO efficiency and safety, this substance is widely used for optical clearing in the mixtures with different OCAs [64, 118, 143-146, 152, 153, 156].

Ethanol is also a solvent that modifies the skin barrier properties. Under sufficiently high concentration (~40%) ethanol facilitates the formation of pores and essentially increases the transport of agents through them due to increasing the size and/or the density of pores in the epidermal membrane [150].

The mechanism of enhancing the permeability of the epidermis *stratum corneum* under the action of propylene glycol is the solvation of keratin in the process of water replacement in the binding hydrogen groups and the inclusion of propylene glycol into the polar heads of the lipid bilayer [151]. It was shown that the mixtures of different OCAs with propylene glycol increase the efficiency of the optical clearing effect, however, the clearing effect induced by propylene glycol itself is weaker than for these mixed OCAs [147].

The last of the diffusion enhancers mentioned above,

Thiazone, is an innovative agent that increases the skin permeability nearly three folds stronger than Azon [36]. It is also more efficient as compared to propylene glycol [36, 147].

To increase the tissue permeability for OCAs one can use combinations of the abovementioned agents. For example, in Ref. [157] for the optical clearing of skin the combined OCA was used, including glycerol, PEG-300, ethanol and DMSO.

Beside the chemical agents, a number of physical methods of surmounting the skin barrier are proposed to enhance the diffusion, including low- and high-intensity irradiation [130, 158], fractional lamp [131, 133] and laser [132] microablation, mechanical microperforation [137, 138], ultrasonic (US) irradiation [134, 135, 159], electrophoresis [160], needleless injection [161], mechanical removal of the surface layer by means of abrasive paper [136], epidermal stripping [162], and microdermabrasion [163].

Different radiation sources (e.g., CO₂ and Nd:YAG lasers, operating at the wavelengths 532 and 1064 nm, respectively, broad-band sources of intense pulsed light, operating in the ranges 650–1200, 525–1200, and 470–1400 nm) have been used to irradiate the skin *in vivo* before the OCA application with different doses and regimes of exposure. The measurements of reflection spectra before and after the exposure have shown that the radiation of Nd:YAG laser in the modes of Q-modulation and long pulses can induce the improvement of transepidermal penetration of OCA by 8-9 folds as compared to the intact skin [130]. In the other study Stumpp et al. [158] used a diode laser, operating at the wavelength 980 nm for the rodent skin irradiation using artificially absorbing substrates on the surface. The laser radiation provided heating of the *stratum corneum* and caused the failure of the protective barrier function. After the removal of absorbing substrates the skin surface was subjected to glycerol application. The OCT study of skin in the exposed regions has shown the increase of the light penetration depth by up to 42%.

The similar principle was used to create regions of microablation under the action of a broad-band flash lamp. The transparent mask with a set of absorbing carbon centres was applied to the skin surface in order to create the regions of epidermis microdefects under the light absorption [131, 133]. Increasing the skin transparency and image clearness of the tattoo, located under the skin at the depth of 300-400 µm, was demonstrated by Genina et al. [133] and Bashkatov et al. [164]. Fig. 3 presents the tattooed skin images before and after the action of the epidermis fractional thermal ablation and 88% glycerol solution.

Fractional laser microablation of skin surface by means of the erbium laser (2940 nm) with the pulse energy from 0.5 to 3.0 J is also an efficient tool of overcoming the barrier for hydrophilic and hydrophobic OCAs [132].

The integrity of the epidermis *stratum corneum* can be disturbed mechanically using a roller with multiple needles. Yoon et al. [137] used this device, applied in

cosmetology, to create transdermal microchannels in samples of porcine skin *ex vivo* with the aim of improving the glycerol penetration into the depth of the skin. The combination of multiple needle perforation with low-frequency ultrasonic effect allowed the increase of the diffusion rate of the 70% glycerol solution into the skin by 2.3 folds in comparison with using the multiple-needle roller solely [138].

The low-frequency US effect (sonophoresis) is one of the noninvasive methods of improving the clearing effect for many OCAs, increasing the depth and rate of their delivery into tissues [134, 135, 165-168]. The combined use of OCA and US provided considerable increase of the depth and contrast of the OCT images of porcine skin *in vitro* and human skin *in vivo* [166]. Xu et al. [134] used the ultrasound with the frequency 1 MHz to enhance the penetration of 60% glycerol solution and 60% PEG-200 solution into the porcine skin *in vitro*. The OCT study has shown the increase of the imaging depth by 40% and 93%, respectively, as compared to using OCA without US.

Fig. 3 Images of tattooed skin surface: a) the tattooed sample before the glycerol action; b) the tattooed sample after the surface microperforation and glycerol impact during 24 hours [133].

Cavitation is the main mechanism of the sonophoresis that explains the tissue permeability increase [159, 167]. The theoretical analysis of surface interaction of the cavitation bubbles with lipid bilayers of the epidermal *stratum corneum* considers three

interaction modes, namely, the shock wave propagation, the penetration of microflux into the *stratum corneum*, and the impact of the microflux on the *stratum corneum* [159]. In the process of growth the bubbles can merge, forming larger bubbles that continue growing creating channels. The channels formed in the *stratum corneum* become larger with time in the direction towards the boundaries, which finally leads to the appearance of inner transport ways [167]. These processes induce disorder in the lipid bilayers of the epidermal *stratum corneum*, weaken the barrier function, and enhance the OCA penetration into the inner layers of skin [168].

The transdermal electrophoresis is a well-known and widely used method of controlled delivery of drugs through the skin aided by low-intensity electric current. The specific features of this method are the following: 1) the transport can be significantly intensified as compared to the passive diffusion; 2) the delivery rate may be actively controlled by modulating the electric current density, which allows individual dosage [169].

The electrophoresis can be applied not only with polar electrically charged agents. The dependence of electroosmotic diffusion upon pH of the solution and the content of NaCl ions in it was studied in Ref. [160]. In the experiments the solution containing 0.07M of NaCl and 0.13M of D-mannitol in 5mM of citric-acid buffer with pH = 6 was used. Compared to the passive diffusion, the volume flux of the agent gradually increased approximately by 20 folds after switching the current on, approaching a constant value. After switching the current off, the volume flux slowly decreased to the values, exceeding those before the beginning of the electrophoresis [160].

Stumpp et al. [161] proposed to use a pistol for needleless injection of glycerol with concentrations of 100, 50, and 25% into the porcine skin *ex vivo*.

The removal of the skin surface layer by slight rubbing with an abrasive paper also helps to enhance the OCA penetration through the epidermis *stratum corneum* and to improve the optical clearing effect. The abrasive paper with the gritness 220 was used by Stumpp et al. [136] for smooth rubbing of glycerol or dextrose solution into the depilated hamster skin *in vivo*. After 2-4 min of rubbing in, excluding any visible damage, the skin gradually became more transparent, allowing the observation of subcutaneous blood vessels. The quantitative analysis of the OCT signals has shown that the light penetration depth increased by 36-43%.

The mechanical removal of outer cell layers of the epidermis can be also implemented via a sequence of surface epidermal strips. The procedure of gluing strips of adhesive tape to the skin surface and tearing them off repeated up to 30 times increases the permeability of skin without its essential damage [162]. In Ref. [139] the authors used slides with cyanoacrylate applied to the skin with moderate pressure, kept nearly 3 minutes, and then were left for 2 minutes more. When tearing the slide off the outer layer of the epidermis was removed. The procedure was repeated 3-6 times till the skin became shining. Then the OCA was applied to the

processed surface under slight pressure that also improved the agent penetration into the skin.

The combined use of chemical and physical OCA diffusion enhancers facilitates further increase of the optical clearing efficiency [118, 148, 149]. Thus, Xu et al. [148] showed that the sodium lauryl sulphate, a surface-active agent often used as an enhancer of penetration of pharmaceutical and cosmetic products into skin, in combination with the US demonstrates a synergetic enhancing effect on the penetration of 60% glycerol solution into the skin. As a result the optical transmission and OCT probing depth increased and the time of reaching the maximal clearing decreased in comparison with the US used solely.

The US processing was also used in combination with Thiazone to improve the penetration of PEG-400 into the skin [149]. It was shown that after the complex processing with Thiazone, PEG-400, and US the diffuse reflection coefficient decreased by 33.7 folds as compared to the control measurements (without clearing). Using only PEG-400 or PEG-400 with Thiazone the reduction of the reflection coefficient was by 2.7 and 3.3 folds, respectively. The probing depth increased by 41.3% in comparison with the control samples.

Fig. 4 The difference between the averaged coefficients of total light attenuation by the intact rat skin *in vivo*, calculated from the OCT data. The column “control” corresponds to the data, obtained before the OCA action. “OCA” – 20-minute topical action of OCA (glycerol-PEG-400 mixture in equal proportions); “DMSO-OCA” – 20-minute action of DMSO-OCA mixture; “US-OCA” – 4-minute action of low-frequency ultrasound and OCA; “US-DMSO-OCA” – combined 4-minute action of ultrasound and the DMSO-OCA (from the data of Ref. [118]).

The comparison of the coefficients of total light attenuation by the rat skin *in vivo* using physical and chemical penetration enhancers separately and in combinations was carried out in Ref. [118]. Fig. 4 presents the result of calculating the value

$$\Delta\mu_t = \frac{\mu_{t(\text{treated})} - \mu_{t(\text{control})}}{\mu_{t(\text{control})}} \times 100\%$$

of the relative attenuation coefficient change as a result of the multimodal skin processing with respect to the control measurements. In this case the control group consisted of animals not subjected to the treatment. For this group $\Delta^{\mu_t} = 0$. The results allow the assessment of the efficiency of different OCA diffusion enhancers (the OCA was a mixture of equal parts of glycerol and PEG-400). It is seen that the OCA column has the minimal height, which means the minimal efficiency of the 20-minute OCA action on the surface of intact skin (the optical clearing is absent). The 20-minute application of 9% DMSO solution in OCA appeared to be less efficient than the 4-minute ultrasonophoresis. However, the maximal efficiency of optical clearing was observed under the combined use of US-DMSO-OCA during 4 minutes.

The combined use of surface epidermis stripping with chemical enhancers of diffusion, such as Thiazone, Azon and propylene glycol, was studied in the rat skin *in vivo* [170]. After tape strips from the skin surface the mixture of the enhancer with PEG-400 was applied to the prepared region. As a result the skin diffuse reflection coefficient was reduced, the greatest change being observed when using Thiazone-OCA, then, in descending order, Azon-OCA, propylene glycol-OCA and OCA without adding the permeability enhancer. These results differ from the data of *in vitro* studies of porcine skin, for which the optimal combination of OCA and enhancer was propylene glycol-OCA, and the minimal reduction of the reflection coefficient was demonstrated by Azon-OCA [147].

The influence of microdermabrasion on the skin permeability for hydrophilic and lipophilic agents was studied in Ref. [163]. Microdermabrasion is a partial ablation and homogenisation of the epidermal *stratum corneum* under the action of high-pressure flow of microparticles. Depending on the pressure (15-25 centimetres of mercury) and the time of exposure the skin permeability for hydrophilic agents increased by 8-24 folds, as compared to the intact skin. This method can be used for enhancing the OCA diffusion as well.

3 Compression clearing

The influence of external mechanical compression on the optical properties of biological tissues *in vivo* is interesting for several reasons. First, the specific features of light propagation in the tissue vary depending on its morphologic, biochemical, and physiological characteristics, therefore, the spectral composition of light, outgoing from the tissue, carries information on its morphologic and functional condition [171, 172]. Second, as a result of local mechanical compression, produced, e.g., by the end of a fibre-optical probe (the area of the force application about a few square millimetres) at the compression site a gradient of the refractive index is induced, and this volume of the tissue plays the role of a lens for the probing radiation, propagating through the tissue. When the mechanical compression is applied to a relatively large area of the tissue surface (of the order of a few

cm²), the lens effects are absent, but the compaction of the medium can manifest itself in the change of its optical characteristics due to the variation of the packing density of the scatterers.

Since the external mechanical compression can essentially change the optical and structural properties of the tissue, this method can be considered as an alternative to the widely used immersion method of controlling the tissue optical parameters, based on the influence of chemical agents. As discussed above, the main mechanisms of the immersion clearing are the transport of water from the tissue and the partial replacement of intercellular or intracellular water with a chemical agent, as a result of which the difference of refractive indices of the scatterers and the interstitial fluid becomes smaller, reducing the scattering. Analogous processes partially occur under the compression, since the removal of water from the intercellular space and the increase of protein concentration in it as a result of applying the external compression should also lead to the reduction of scattering.

The effect of increasing the depth of laser radiation penetration into a biological tissue under the application of the external local mechanical pressure was demonstrated more than 30 years ago [173]. From that time a considerable number of publications appeared, related to using the influence of external mechanical compression of tissues on their optical properties (absorption and scattering).

The first studies [174, 175] were carried out with the tissue samples *ex vivo*. The authors noticed that the external compression changed the optical properties of the tissue samples, which manifested itself in the change of diffuse reflection and transmission of light by the samples of tissues. Thus, in Ref. [174] the studies of compression of soft tissue samples demonstrated the growth of the transmission coefficient and the reduction of the diffuse reflection coefficient in the spectral region from 400 to 1800 nm. It was concluded that the compression increases the coefficients of absorption and scattering of the tissue, and the possible mechanisms of the “clearing” are the reduction of the sample thickness and its dehydration. Similar results were also obtained by the authors of Ref. [175]. The studies of the compression effect on the samples of porcine skin have shown that the mechanical compression causes the increase of the light transmission through the samples, and the effect is inertial.

The mechanical compression also changes the optical properties of tissues *in vivo*, which is accompanied by the spectral changes both in the diffuse reflection [176-186] and in the fluorescence [185, 187-189]. The authors of [176, 187] were one of the first researchers who noticed the influence of the compression on the spectra of diffuse reflection and autofluorescence of human skin *in vivo*. It was reported that the pressure exerted on the skin reduces the depth of the dip in the green region of the spectrum that indicates the presence of blood in the tissue, as a result

of which the reflection coefficient of skin in this region increases, while in the red and yellow region it decreases. In the reflection spectrum an isobestic point was found, for which the reflection coefficient does not change in the presence or in the absence of compression [176]. The external compression also caused the increase of the skin autofluorescence intensity in the short-wave region of the visible spectrum [187].

The reduction of diffuse reflection in the region 1100 – 1700 nm was reported [178] in human skin *in vivo*. It was shown that under the increase of the pressure of the fibre-optical probe on the skin surface the diffuse reflection is decreased. The authors related such behaviour with the change of the internal structure of the skin tissue. A similar fact was also noticed in the visible region of the spectrum [184].

In Ref. [185] the influence of short-time (smaller than 2 seconds) and long-time (greater than 30 seconds) mechanical action upon the diffuse reflection and autofluorescence spectra of human skin *in vivo* was studied. It was shown that at high pressure the significant spectral changes occur both for short-time and long-time compression, the probe pressure affecting not only the optical, but also the physiological parameters of the skin.

The influence of external compression on the physiological parameters of breast was studied by the authors of Ref. [180]. Under the compression of the tissue the light scattering was reduced, also decreased was the total blood content and the saturation of the tissue with oxygen. After the compression removal the effect of hyperaemia was observed. The changes in the blood circulation system (compression of blood vessels and reduction of blood oxygenation), to the opinion of the authors of Ref. [181], can enhance the sensitivity and specificity of early diagnostics of cancer.

At present the external mechanical compression is used as a method that allows the increase of image resolution and contrast in optical coherence tomography (OCT) and microscopy [28, 190-193].

The method of compression optical clearing of tissues is implemented instrumentally (tissue optical clearing device, TOCD) [29, 31, 194-196].

Finally, the mechanical compression of skin allows the assessment of the content of chromophores in it, whose absorption under the normal conditions is masked by the absorption by other chromophores. Thus, the extrusion of blood from the region of compression makes it possible to evaluation the content of carotenoids in the skin by the spectra of diffuse reflection [197], and the content of melanin in skin by the spectra of fluorescence [171, 172].

Although the amount of publications related to the mechanical compression of tissues is relatively large, the results are often somewhat controversial, which is mainly due to the difference in the conditions of the force application (local or distributed) and the detection of the reflected light (fibre-optical or open detecting system) [198]. Besides, it is necessary to take the inertial character of the tissue response to the external

action into account.

The compression method of controlling optical parameters has a number of potential advantages in comparison with the immersion method. For example, moderate mechanical compression saves the barrier functions of the *stratum corneum* and the epidermis as a whole.

In the visible region the absorption coefficient of water is insignificant as compared to the near IR region, where the absorption peaks caused by the combinations and overtones of fundamental vibrations are located. In the skin the absorption spectral bands of water bound with the proteins of interstitial matrix are shifted with respect to the bands of free water towards the long-wave region of the spectrum. Thus, the manifestations of bands in the reflection spectrum of skin at 1160 nm and 1220 nm were reported [199], attributed by the authors to the absorption bands of free and bound water. The absorption bands at 1879 nm and 1890 nm were attributed [200] to the absorption of free water, and the bands at 1909 nm and 1927 nm to the bound water.

The effect of water transport was studied by Rylander et al. [196]. In the skin the natural gradient of water content depending on the depth exists. In the *stratum corneum* the water content depends upon the air humidity and can be at the level 15%. The water content grows with depth, reaching 70% in dermis. A considerable part of this amount is free water, the molecules of which due to their high permeability percolate from the region of external compression into the adjacent regions. The water migration from the skin regions subject to compression leads to the matching of the refractive indices of the tissue components [31], which reduces the scattering coefficient.

The water transport in the skin tissue can be analysed on the base of two-phase nonlinear mixture [201], according to which the skin is presented as a solid elastic matrix, formed mainly by elastic fibrils and cells and filled with water. This model is sufficient to present the composition and mechanical properties of the skin well enough [196, 201]. Basing on this model the authors of Ref. [193] studied the influence of mechanical compression on the optical properties of the skin. The samples of porcine skin *ex vivo* and human skin *in vivo* were studied using OCT at the wavelength $\lambda = 1310$ nm. It was found that for the porcine skin samples *ex vivo* the compression reduces the water fraction in the tissue by more than three folds (from 0.66 to 0.2).

The detailed analysis of possible mechanisms affecting the skin optical properties was carried out in Ref. [199] where the influence of human skin compression on the variation of its scattering properties and the free and bound water content were studied. The changes in the diffuse reflection spectra were correlated with the effects of tissue deformation within the framework of two-phase nonlinear mixture model. The optical properties of the skin were considered basing on the three-layer medium (epidermis, dermis, subcutaneous fat), and the scattering properties of the

tissue were determined as the combination of scattering by large (Mie scattering) and small (Rayleigh scattering) particles. The expressions for the scattering coefficients of each of the skin layers were taken from Refs. [202, 203], and the skin absorption in the near IR region was determined as the superposition of absorption by water and lipids. The water contribution into the absorption amounted to 20% for epidermis, 70% for dermis, and 60% for subcutaneous tissue (fat and muscle) [203, 204].

Experimental diffuse reflection spectra were analysed for two absorption lines of water in the near IR region, 1160 nm and 1220 nm, attributed by the authors to the absorption of free and bound water, respectively, basing on the comparative analysis of the reflection spectra of skin and pure water.

The measurement of water content in the skin *in vitro* has shown that under compression the intensity of the band at 1220 nm significantly grows, while the intensity of the band at 1160 nm decreases. When the pressure is increased to 376 kPa, the absorption peak at 1160 nm almost vanishes. This indicates the fact that under the compression of skin the free water leaves the compressed region, while the bound water that forms complexes with proteins stays. When the pressure achieves 400 kPa, the volume of free water decreases to 30% of its initial value. This is the main cause of the tissue deformation. Under the long-time compression the absorption peak of free water decreases with time, and the peak of bound water increases. At the early stages of the deformation due to the large stress in the tissue the free water leaves the compressed region with sufficiently high rate. As the stress relaxes, the deformation of the tissue and the rate of free water migration gradually decrease. After nearly 6 minutes the tissue deformation and the water transport stop. The duration of this process is also related to the compression magnitude.

Thus, for the analysis of the structural and optical changes occurring in the skin *in vivo* under the conditions of external compression the above model of the tissue can be considered, according to which the skin consists of a solid matrix formed by the collagen fibres and the interstitial fluid, the major part of which is water. When the skin is compressed, the solid matrix is deformed and the interstitial fluid leaves the region subject to compression with the rate determined by the density of packing of the matrix fibres and the viscosity of the fluid. This hypothesis is in good agreement with the experimental data. The relation of skin deformation and relaxation time under the conditions of compression depends upon the elastic properties of the fibrils and the density of their packing, as well as upon the amount and viscosity of the fluid in the tissue. Since the bound water is integrated in the tissue solid matrix, the measurements of free and bound water content variations can reflect the deformation of the solid matrix and the transport of free water in the tissue under the conditions of compression.

4 Photochemical and photothermal clearing

For a number of applications, particularly, in the case of laser therapeutic or surgical interventions, one can control the optical properties of the tissues by means of photochemical or photothermal action. The heating and coagulation of a tissue change its optical properties, and this should be taken into account in the calculation of the radiation dose in the process of treatment [3, 22]. The method of controlling optical properties of fat tissue, which is usually a strongly scattering tissue, was proposed in [205, 206]. The method is based on the combined photochemical and photothermal effect [2, 207] induced in the fat tissue cells.

The selective photothermal action on the fat tissue can be implemented by the laser radiation wavelength choice using the absorption by the endogenous (own) tissue chromophores, in the present case the lipids contained in the fat drop of adipocytes, under the exposure to light with the wavelength 1210 nm [208]. However, with exogenous chromophores, e.g., the indocyanine green (ICG), the efficiency of light interaction with tissue may be essentially higher and more selective [209-213]. The intensity and position of ICG absorption peaks depend upon the solvents used [214-216]. The solutions with complex compositions, e.g., such as water-alcohol-glycerol, stabilise the ICG absorption peaks [215]. Besides that, the alcohol component of the solution makes the cell membrane permeable for the dye [217-219]. The next step of the interaction between the dye and the cell is related to the light action. The light causes photochemical reactions; for ICG they simultaneously follow two scenarios: 1) photodynamic reactions; 2) reactions yielding toxic products [216]. Depending on the intensity, the biological response of the cell may lead to reversible or irreversible injury of the cell membrane. Reversible membrane injury consists in the creation of new pores or enlargement of the already existing ones, which facilitates efficient exchange between the cell content and the environment. In fat cells the presence of pores increases the lipolysis, as a result of which the intercellular space becomes filled with the cell content and decay products (triglycerides, fatty acids, water and glycerol) [37, 220-223]. The appearance of such immersion fluid in the intercellular space facilitates the process of optical clearing of the adipose tissue [37].

The probability of triglyceride leaving the cell through the membrane pores is rather small due to the relation between the pore diameter (0.1 – 2 nm) and the triglyceride molecule size (nearly 1-2 nm) [221-223]. The hydrolysis of triglycerides occurs in two stages. At the first stage the hydrolysis of external complex ether bonds occurs; this process is catalysed by the lipase enzyme. The hydrolysis of triglycerides is referred to as lipolysis. The monoglyceride produced at the first stage of the triglyceride decay, is further hydrolysed by the nonspecific esterase, producing glycerol and three molecules of higher fatty acids [221-223]. The

hydrolysis products can easily leave the cell through the pores, since their molecular size is small compared to the pore size. In this process the refractive index of the intercellular fluid ($n_m = 1.36$) [45] approaches the refractive index inside the adipose cell itself ($n_b = 1.44$), the medium becomes more optically homogeneous and, as a result, more transparent [22].

In Refs. [37, 220] the photodynamic effect in adipose cells *in vitro* sensitised with brilliant green (BG) was studied. The result was also the cell lipolysis. The hypothesis of pore formation is in good agreement with the experimental results [37, 206, 220, 224].

Fig. 5 presents the transmitted light images of the subcutaneous fatty tissue sample, stained with BG, before (a) and after (b-e) the irradiation with a dental diode lamp Ultra Lume Led5 with the wavelengths 442 and 597 nm and the total power density 75 mW/cm² during 15 minutes at the constant temperature 33°C. The cell lipolysis is seen to stimulate the leakage of a part of intracellular fluid into the intercellular space and the tissue gradually undergoes immersion clearing.

5 Applications of optical clearing

5.1 Optical coherence tomography

OCT is a noninvasive method of visualising the internal structure of optically inhomogeneous objects that allows the study of tissue internal microstructure with high resolution and without disturbing its integrity [18]. However, the multiple scattering essentially worsens the OCT imaging characteristics, namely, the image resolution, probing depth, and the precision of localisation [21]. In this connection the progress of optical clearing techniques is of huge potentiality for noninvasive OCT medical diagnostics [28, 29, 35, 36, 39, 190, 192, 196].

The efficiency of mechanical compression for better differentiation of chronic inflammation and carcinoma in the OCT images was studied by Agrba et al. [28]. They also determined the threshold impact that allows the detection of difference between these cases. Using the endoscopic probe of unique construction that allowed the control of the compression force applied to the tissue, the possibility of differentiating the pathologic changes of rectum *ex vivo* with inflammation and carcinoma was demonstrated.

The same research team has shown that the application of compression to human skin causes various changes of the optical properties of skin layers depending on their elasticity. These changes increase the contrast of the OCT-imaged boundaries between the layers [192].

The efficiency of the mechanical device for biotissue optical clearing, designed by Rylander et al. [196] was determined using OCT [29]. The authors have shown that the depth of light penetration into the human skin *in vivo* at the OCT wavelengths 850 and 1310 nm increase by 3 folds in the area of compression. The contrast of

the OCT image of the epidermis-dermis boundary was also increased. OCT allowed the specification of compression clearing mechanism. Thus, the OCT M-scans recorded during the pressure application have shown that the optical penetration depth monotonically increases, undergoing a jump at the initial time period (5-10 s), during which the water content in the tissue abruptly decreases and the group refractive index increases in the local area of pressure application.

The authors of Ref. [191] proposed to use OCT with compression for continuous noninvasive monitoring of blood glucose content. The results of *in vivo* skin studies at different pressures have shown that the pressure of the OCT probe on the skin reduces the artefacts related to the object motion, but may shift the OCT signal slope that allows the blood glucose monitoring. Thus, the controlled external pressure < 1 kPa significantly improved the precision and reproducibility of the glucose OCT monitoring.

The potentialities of immersion optical clearing are mainly related to the fact that OCT allows the imaging of relatively thin layers of tissues that can be rapidly impregnated with OCA applied to the surface. In Refs. [21, 22] it was demonstrated that the internal tissues, such as the walls of blood vessels, oesophagus, ventricle, large intestine, and other organs can be imaged to the depth of 1-2 mm. The application of immersion liquids increases the probing depth nearly by 3.5 folds and significantly improves the image contrast of the internal inhomogeneities [225, 226]. The possibility of malignant melanoma diagnostics, observation of subepidermal cavity, and control of the skin scattering properties under the application of glycerol and propylene glycol to its surface was shown by the example of OCT visualisation of human skin *in vitro* and *in vivo* [22]. Moreover, the variation rate of the OCT A-scan slope in the course of the optical clearing allows additional differentiation of the healthy and tumour tissue of the human oesophagus and mammary gland [103-105, 227, 228].

Optical coherence tomography offers a unique possibility to assess the variation of the tissue light scattering coefficient with sufficient depth resolution. The total light attenuation coefficient μ_t of the tissue local volume, which is a sum of the absorption coefficient μ_a and the scattering coefficient μ_s , may be found by fitting the parameters of the approximating curve, calculated within the framework of the appropriate model, using the local A-scan slope of the OCT signal [18, 118, 230-232].

The single scattering model is based on the assumption that only the light undergoing single scattering (ballistic photons) preserves the coherence properties and contributes to the OCT signal. In this case the OCT signal $i(z)$ is determined as [18, 230, 231]: $(\langle i^2(z) \rangle)^{1/2} \approx (\langle i^2 \rangle_0)^{1/2} (\exp(-2\mu_t z))^{1/2}$, where z is the depth, from which the reflected signal comes.

Fig. 5 Transmitted light images of a subcutaneous fatty tissue sample stained with brilliant green before (a) and after (b–e) light exposure during 15 min. The time interval between the image recording and the end of exposure is 8 min (b), 19 min (c), 39 min (d), 120 min (e). The sample temperature is 33°C. Diode lamp, 442 and 597 nm, the total power density 75 mW/cm².

It is known that the result of OCT is the measurement of the reflected signal intensity from the studied tissue, $R(z) \propto \left(\langle i^2(z) \rangle \right)^{1/2}$, upon the depth z . The OCT signal intensity depends upon the reflectance $\alpha(z)$ of the tissue at the given depth and the total attenuation coefficient $\mu_t = \mu_a + \mu_s$ of the tissue. In accordance with the single scattering model that is valid both for weakly scattering tissues and for the surface layers of strongly scattering tissues [231], the reflected power is proportional to $\exp(-\mu_t z)$ [18], i.e., can be approximated with the expression [118]: $R(z) = A \exp(-\mu_t z) + B$, where A is the coefficient equal to $P_0 \alpha(z)$, with P_0 being the optical power of the beam, incident on the tissue surface, $\alpha(z)$ being determined by the local capability of the tissue to scatter the light in the backward direction (to reflect), which is largely dependent upon the local value of the refractive index, and B being the background signal.

Since the absorption coefficient is essentially smaller than the scattering coefficient for many tissues in the near IR spectral range, the exponential decay of the ballistic photons is mainly determined by the scattering coefficient [18]. The scattering coefficient depends on the difference of refractive indices in the tissue volume, so that the increase of the refractive index of the interstitial fluid and the corresponding reduction of scattering is recorded as the change (decrease) of the slope of the OCT signal amplitude as a function of the probing depth [18, 35, 89, 90, 93, 95–100].

This method allows the measurement of the permeability coefficient of tissues both for OCAs and for the medicinal preparations, possessing the properties of OCA, using two approaches: measuring the OCT signal slope (OCTS) and the OCT signal amplitude (OCTA) (Fig. 6a) [35, 95, 232]. In the OCTS approach, analysing the variation of the OCT signal slope due to the OCA diffusion, one can calculate the mean permeability coefficient of a certain region in the tissue. In this case two-dimensional OCT images are averaged over the lateral coordinate (x axis) to get a one-

dimensional depth distribution of the OCT signal intensity. In the tissue it is necessary to choose the region, for which the signal is linear and its fluctuations are minimal, where the tissue thickness (z_{region}) must be measured. Then the monitoring of the agent diffusion in the chosen region is performed and the time of diffusion (t_{region}) is recorded. The mean permeability coefficient ($\bar{P}$) can be calculated as the thickness of the region divided by the time, during which the diffusion of the agent through the chosen region occurs $\left(\bar{P} = \frac{z_{\text{region}}}{t_{\text{region}}} \right)$ [232].

The OCTA approach can be used to calculate the permeability coefficient at a definite depth in the tissue: $P(z) = z_i / t_{z_i}$, where z_i is the measurement depth and t_{z_i} is the time of agent diffusion to the given depth. The value of t_{z_i} should be calculated from the time of the agent application to the time of the beginning of the OCT signal amplitude variation, caused by the OCA [232].

These approaches allowed the assessment of the following permeability coefficients: rabbit cornea for mannitol $(8.99 \pm 1.43) \times 10^{-6}$ cm/s [95], rabbit sclera for mannitol and 20% glucose solution $(6.18 \pm 1.08) \times 10^{-6}$ and $(8.64 \pm 1.12) \times 10^{-6}$ cm/s, respectively, [95], human sclera for corticosteroid $(2.40 \pm 0.32) \times 10^{-5}$ cm/s (Fig. 6b) [93], porcine aorta for 20% glucose solution $(1.43 \pm 0.24) \times 10^{-5}$ cm/s [99], epidermis and dermis for 40% glucose solution $(6.01 \pm 0.37) \times 10^{-6}$ cm/s and $(2.84 \pm 0.68) \times 10^{-5}$ cm/s, respectively, [97], lung tissue for 30% glucose solution $(1.35 \pm 0.13) \times 10^{-5}$ cm/s (the norm) [100], and lung tissue for 30% glucose solution in the case of different malignant neoplasms [100].

The depth-resolved analysis of the tissue optical properties using OCT allows the reconstruction of two-dimensional diffusion maps. The visual representation of the molecular diffusion front was first demonstrated by Ghosn et al. [97] in the course of noninvasive determination of the penetration rate of 20% glucose

solution into the rhesus macaque skin *in vivo*.

Fig. 6 The region and the depth, indicated in the averaged OCT signal, used in OCTS and OCTA approaches (a), and the time dependence of the OCT signal slope in the process of interaction of the sclera with the corticosteroid solution (b) (from the data of [93]).

Using the OCT methods one can reveal the variations of the effective refractive index of the adipose tissue as a result of the light photodynamic action, which arise both directly after the exposure, and in the process of the system biological response [38, 224]. The observed changes of effective refractive index can be interpreted as the reduction of the relative refractive index of the scatterers, which can be related to the immersion optical clearing.

Fig. 7 shows the series of OCT images of adipose tissue stained with BG before (a) and after (b-d) the irradiation with the light of a diode lamp during 15 minutes and under the heating of the samples to the physiological temperature. Fig. 7,b shows the image of the adipose tissue immediately after the irradiation, while the OCT images in Figs. 7,c and 7,d correspond to relatively long observation time (60 and 120 min., respectively). The mean thickness of the samples amounted to $237 \pm 10 \mu\text{m}$.

The visual comparison of the OCT tomograms of the adipose tissue shows that after the irradiation the tissue

structure considerably varies in time. One can easily see the changes of the outer layer of the cellular structure that becomes more homogeneous, which can be associated with partial destruction of cells and formation of immersion layer consisting of intracellular content and the products of the adipose component hydrolysis. In Fig. 7(b-d) this layer is as thick as 80-90 μm. The mean size of adipocytes in the tissue amounts to 60-70 μm.

Provided that the geometric thickness of the studied layer is known, the effective refractive index n is calculated as $n = z/l$, where l is the geometric path length (the true sample thickness in μm), and z is the layer thickness observed in the OCT image, i.e., the optical path length in μm. The geometric thickness was measured using a micrometre gauge. The optical path length was found from the OCT signal as the difference between the depths of two peaks, corresponding to the sample boundaries (see Fig. 7). For better determination of the boundaries the A-scans were averaged over the lateral region of 2 mm. This operation smooths out the stochastic noise and the disordered cell structure of the tissue, while the peaks, corresponding to the sample boundaries, become more distinct. The calculation of the refractive index showed its monotonic decrease with the growth of observation time.

The observed changes in the OCT images can be interpreted as the result of lipolysis and cell destruction at the surface of the sample due to the photochemical effect. The intracellular fluid leaks from the cells and fills the intercellular space, as a rule, containing the intercellular fluid, thus producing the cleared (more homogeneous) outer layer and giving rise to optical clearing inside the tissue by matching the refractive indices of cells and intercellular medium.

In Ref. [233] the chemically induced lipolysis activity was assayed by measuring the amount of glycerol released from the cells into the surrounding medium. Besides, using frame-by-frame CARS (coherent anti-Stokes Raman spectroscopy) processing of a single living cell, the authors could control the morphological changes of lipid droplets in the cells. They found that the microscopic lipid droplets appeared gradually in the course of the experiment, achieving the size of nearly 1 μm in 60 min after the impact. All these observations allow the assumption that analogous processes may occur in the case of light-induced lipolysis.

The results of OCT measurements of the refractive index of adipose tissue after the exposure to light can be explained basing on the idea of forming two layers with the course of time. One of these layers is cleared (the released intracellular fluid) and the other one is scattering (cells). The refractive index m of the scattering medium can be expressed as [128]

$$m \equiv n_0 + \Delta m = \bar{n} + \frac{\bar{n}^2 - \bar{n}^2}{\bar{n}} Q(\lambda/l), \quad (1)$$

Fig. 7 OCT images of adipose tissue stained with BG before the irradiation (a), immediately after 15-minute irradiation (b), in 60 min (c), and in 120 min (d) after the irradiation. The source was the dental diode lamp Ultra Lume Led5 (442 and 597 nm, 75 mW/cm²). The dye concentration is 6 mg/ml. The sample temperature is 37 °C. The curves correspond to A-scans of the OCT image, averaged over the whole B-scan region [224].

where

$$\bar{n} = n_0 \left[1 + \left(\frac{n}{n_0} - 1 \right) \frac{V_s}{V_0} \right], \quad (2)$$

V_s is the total volume of scattering particles, V_0 is the volume of the scattering medium, $\bar{n}^2$ is the mean square value of the refractive index fluctuations, $Q(\lambda/l)$ depends upon the shape and aggregation of scatterers, and l is the correlation length of randomly distributed fluctuations of the refractive index. $Q = 1.17$ for the limit case of large correlation length, $l \gg \lambda$ (large particles). In the process of the adipose tissue degradation the two-phase system appears, consisting of cytoplasm of the adipose cells (the cleared outer layer in Fig. 7,b) and the adipose cells themselves. Each phase of the medium has its own thickness and refractive index. Denoting the time-dependent thickness of the layer of adipose cells as $H(t)$, and the thickness of the outer layer of transparent cytoplasm as $[L - H(t)]$, the mean refractive index of the layer with the thickness L , containing two layer with different refractive indices, can be written as

$$m_T = \frac{[L - H(t)]}{L} \bar{n} + \frac{H(t)}{L} m, \quad (3)$$

where m and $\bar{n}$ are defined by Eqs. (1) and (2). Since m is always greater than $\bar{n}$ due to the effect of scattering and tissue degradation, this leads to the decrease of $H(t)$, and the summary refractive index should decrease with time.

In the process of adipose tissue degradation and formation of greater amount of lipolysis products the conditions of refractive index matching can be implemented, which will lead to further reduction of light scattering, i.e., the part of the refractive index m , depending on these processes. Both these mechanisms, the tissue degradation and the refractive index matching, explain the experimentally observed reduction of the mean refractive index of the sample layer, i.e., the reduction of its scattering capability, in the course of time.

5.2 Optical projection tomography

Optical projection tomography (OPT) is a new approach to three-dimensional visualisation of small biological objects. It fills the gap between the MRT and confocal microscopy (CM), being the most suitable for studying the objects with the size from 1 to 10 mm [234]. The possibility to analyse the organisation of a biological tissue in three dimensions is inestimable for understanding the embryonal development, a complex process in which the tissues undergo a complex sequence of transformations with respect to each other [235]. Since the OPT allows the registration of both the absorption and emission profiles, this method offers an opportunity to use different staining techniques, developed to observe the spatial distribution of gene activity [234, 235]. Other important applications of the OPT help in the analysis of normal and abnormal morphology and in determining the position of labelled cells in the tissue [235].

The most widely used approach to the OPT imaging implies the suspension of the object in the immersion liquid in order to reduce the surface light scattering and

to decrease the refractive index nonuniformity all over the sample. This means that the light passes through the sample along nearly straight trajectories (ballistic photons), and the standard algorithm of back projection can generate images with high resolution [234]. Alanentalo et al. [236, 237] have shown that the OPT allows successful construction of three-dimensional images of specially labelled structures inside the cleared organs of a mouse (see, Fig. 8).

Fig. 8 Single image from the OPT scan datasets with the selected regions of interest (inset) (a) and volume reconstruction of the pancreas based on the background autofluorescence (dark gray) and the signal from insulin-specific antibodies (white-islets) with the regions of interest (inset) (b) [237].

The role of OCA was played by «Murray's clear» (1 part of benzyl alcohol and 2 parts of benzyl benzoate (BABB)) with the refractive index 1.55.

5.3 Fluorescence imaging

The methods of fluorescence spectroscopy and microscopy belong to noninvasive methods of biomedical diagnostics. Fluorescence spectra often provide detailed information about the fluorescent molecules, their conformations, sites of bonding and interaction with cells and tissues [238]. Under the excitation with ultraviolet light ($\lambda \leq 300$ nm) one can observe the autofluorescence of both the proteins and the nucleic acids. The porphyrin molecules are also endogenic phosphors, the content of which in cells considerably increases under some pathologic conditions. Besides, some bacteria, causing, e.g., the inflammation diseases of skin [239] and gums [240], inhabiting the caries tooth lesions [241], can accumulate considerable amounts of porphyrins. Combining the measured autofluorescence spectra with those of reflection and absorption, OCT and other optical methods allows the diagnostics of caries and tooth tissue demineralisation [241-245], skin cancer [246, 247], and pre-cancer changes of cervical tissue [248], mammary gland [249], and other diseases.

Thanks to the growing number of fluorescent dyes, specifically staining the biological molecules (nucleic acids, proteins) or cell organelles, the methods of fluorescence microscopy acquire greater and greater significance [238].

At present in different biomedical studies the confocal microscopy (CM) is widely used, which allows visualisation of internal structure of biological tissues at the cellular and subcellular level [250, 251]. A confocal microscope illuminates a small volume inside the object and detects the scattered light of fluorescence signal from the same small volume. The main CM advantage as compared to the methods of common microscopy is the capability to obtain the optical cross-sections of thick samples [251]. This method allows the creation of high-quality (with micron spatial resolution) images of cell layers. The high contrast and spatial resolution of CM images are due to probing a small volume of the medium, limited by the size of the central focal spot formed by the focusing optical system. The main limitation of CM in the skin studies is the considerable scattering that spoils the quality of cell imaging. The increase of the transparency of the outer layers of skin may improve the light penetration depth, the contrast and the spatial resolution of the CM.

In the publications by Meglinsky et al. [48, 49] basing on the Monte-Carlo modelling the possibility of increasing the human skin probing depth was considered theoretically for the first time using the method of reflectance CM with the reduction of spatial fluctuations of the refractive index of the outer skin layers by optical clearing. Later in the paper by Dickie et al. [83] the experimental method was described that allowed visualisation of microvessels of different mouse tissues using the CM at the depth up to 1500 μm below the sample surface due to the optical clearing of thick slices of the tissue. The OCA for CM in Refs. [252-256] was the commercially available multicomponent agent FocusClear™ [257], especially developed for using in fluorescence and non-fluorescence light microscopy. FocusClear™ does not give rise to the tissue dehydration and allows the improvement of the obtained image quality. The refractive index of this agent is 1.46.

FocusClear™ was successfully used for confocal imaging of murine rectum and ileum [252, 253], the wall of the human ileum [254], the murine pancreas [255], and the brain of the insect (*Diploptera punctata*) [256] *in vitro*. The optical clearing facilitated the identification of spatial and temporal changes of crypt morphology of the murine large intestine with colitis, as well as the detection of transgene fluorescent proteins, expressed in large intestine and ileum [252]. The increase of resolution and visualisation depth of the microstructure and vascular system of ileum was observed under the combined use of blood vessel staining and optical clearing [253]. The authors of Ref. [255] declare that by using OCA the thickness of the studied samples can be increased by 80 folds, which allows better reproduction of the structure and the vascular system of the tissue (see Fig. 9). The use of OCA allowed three-dimensional reconstruction of microvascular architecture of the insect brain with sub-micron resolution to the depth of 1500 μm [256].

Fig. 9 Fly-through presentation of the islet vasculature using multiple projection angles and magnifications. Dimensions of the scanned volume: 521 μm (x) $\times$ 521 μm (y) $\times$ 333 μm (z, depth). Two islets reside in the scanned volume [255].

However, the visualisation depth of 500-1500 μm is not sufficient for three-dimensional reconstruction of neuron networks of the murine brain as a whole. Hama et al. [85] developed an OCA, called *Scale*, which not only increases the transparency of a biological sample, but also does not reduce the intensity of fluorescence signals from the cleared structures.

Dodt et al. [86] used the idea of illuminating the object with so called light sheet, which allows observation of macroscopic objects, such as the brain as a whole, with micrometre resolution. The sample is illuminated from two sides with the laser radiation in the blue part of the spectrum, forming a thin planar light beam. The fluorescence signal is, therefore, emitted only from a thin optical section and is collected by the objective lenses. The declined light is blocked by a filter, and the image is projected through a cylindrical lens onto the screen of a digital camera. Since all sample parts below or above the light sheet are not illuminated, the emission is not excited beyond the limits of the focus and, therefore, is not to be eliminated from the useful signal. This approach is referred to as ultra-microscopy (UM). In comparison with CM or two-photon microscopy, UM can rapidly create optical sectioning of macroscopic samples, since the objectives with small focal power and small numerical aperture can provide a large field of view. However, the use of UM is completely dependent upon the optical transparency of biological objects, so that the improvement of optical clearing efficiency, including that due to the development of new OCAs, remains to be an urgent problem [86, 87, 258-262]. In Ref. [258] it was shown that the use of optical clearing allowed cellular resolution in sectioning the fixed mouse brain and detecting individual neurons, labelled with green fluorescent protein, in the removed murine hippocampus. In the isolated hippocampus the three-

dimensional images of dendrite structures and processes of neuron populations were obtained. The use of UM includes the imaging of cleared murine organs and total embryo specimens, adult insects *Drosophila*, and other fixed tissues with the size of a few millimetres [259].

Ertürk et al. [87] found that tetrahydrofurane (THF) in combination with BABB can completely clear the spinal cord, preserving its fluorescence. Recently Ertürk et al. [262] developed a new protocol of optical clearing using the dibenzyl ether instead of BABB in combination with THF to get three-dimensional images of neurons of the whole mouse brain using the UM. This method was called three-dimensional imaging of solvent-cleared organs (3DISCO). In this study the clearing of the tissue took only three hours, and the visualisation was performed during 45 minutes.

5.4 Photoacoustic imaging

Optothermal effects occur in a tissue due to its interaction with pulsed or intensity-modulated optical radiation. Such interaction gives rise to a number of thermoelastic effects in the tissue, in particular, the generation of acoustic waves. Detection of the acoustic waves is the base of the optoacoustic (or photoacoustic) methods [1, 3]. The main applications of the photoacoustic (PA) method are in the field of visualisation of strongly absorbing objects, such as blood vessels [263-265] and tumours [266]. This method can be also used for imaging of subcutaneous structures [267] and measuring the blood oxygenation [268]. The quality of the PA signal depends upon the penetration depth of the optical signal and the damping of the acoustic signal.

The optical clearing technologies allow the increase of the tissue transparency, which facilitates the reduction of optical radiation scattering and increases the depth of the detected inhomogeneities. On the other hand, the flow of OCA into the tissue and of water out of the tissue changes the composition and structure of the skin, thus changing the acoustic impedance and absorption and, therefore, the acoustic wave damping [269]. Recently it was demonstrated that the optical resolution of PA microscopy can be significantly increased using the aqueous solutions of glycerol as OCA [270]. Liu et al. [115] studied several OCAs *in vitro* and *in vivo*, and showed that PEG-400 in combination with Thiazon improves the visualisation of deep blood vessels of skin, while glycerol is better for imaging small branching blood vessels. In this case the immersion time should be controlled to prevent the reduction of the signal amplification.

Based on the amplification of PA signal at the expense of reduction of scattering, this method can be also efficiently used for noninvasive monitoring of glucose content in blood and tissues, which was demonstrated both in phantoms [271, 272], and in the *in vitro* and *in vivo* studies [273].

By means of PA spectroscopy the influence of glucose can be detected measuring the interval between the peaks of the pressure waves induced by the laser

radiation [272]. The studies demonstrated the applicability of PA spectroscopy to measuring the concentration of glucose [272]. The maximal relative change in the PA response was observed in the region of the second C-H overtone at the wavelength 1126 nm with the additional peak in the region of the second O-H overtone at the wavelength 939 nm [273]. Besides, the analysis of the generated pulsed temporal profile of the PA signal allows the determination of the glucose influence on the tissue scattering, which decreases with the growth of glucose concentration [22, 274].

5.5 Nonlinear and Raman microscopy

Multiphoton excitation of molecules is a nonlinear process, involving the absorption of two or more photons, the total energy of which is sufficient to induce a transition of the molecule to the excited state. Two-photon technique uses the photons with the wavelength of the second harmonic of the incident radiation, coming exactly from the focal region of the incident beam [2, 3]. The unique advantage of the multiphoton microscopy consists in the possibility to study three-dimensional distributions of chromophores, excited by the ultraviolet radiation in thick samples. Such investigation becomes possible because the chromophores can be excited by the radiation of the second harmonic (e.g., at the wavelength of 350 nm), providing high quantum yield under the irradiation of the tissue, e.g., with the laser light having the wavelength near 700 nm, where the tissues possess high transparency. In this case the long-wave radiation can reach deeper layers with less tissue damage [3].

The use of OCA can appear particularly important for improving the two-photon microscopy [23], since it was shown that the light scattering effect strongly reduces the light penetration depth to values, smaller than the depth of the single-photon fluorescence, whereas the resolution remains generally unchanged [251]. This happens mainly because of the exciting beam defocusing in the scattering medium. On the other hand, this method is useful for better understanding of molecular mechanisms of the optical clearing of the tissue under the immersion and dehydration [23].

The improvement of the deep-tissue two-photon signal by means of optical immersion method using hyperosmotic agents, such as glycerol and propylene glycol in the dehydrated form, and the glucose solution, was first demonstrated by Cicchi et al. [23] in the experiments with human skin *ex vivo*. The optical clearing of deeper layers of the tissue occurs mainly at the expense of the cumulative effect of reducing the scattering in the near-surface layers of the tissue sample, which provides smaller damping of the incident and detected radiation of fluorescence. Better focusing (smaller distortion of the focused beam) is achieved in a medium with weaker scattering [23].

Tseng et al. [251] have shown that the combined confocal/two-photon microscopy provides a noninvasive method of microscopic studying of the scaffold structure, which can be a valuable tool for

complex investigation of biomaterials and their interaction with the molecules/cells of interest inside the scaffold. The integration of optical clearing with FocusClear, confocal/two-photon microscopy, and three-dimensional presentation is an efficient approach to the microscopic study of the scaffold structure.

Additional morphological information is provided by combining the second harmonic generation (SHG) microscopy with the microscopy based on two-photon excitation [275].

At present SHG is one of the new high-resolution nonlinear-optical imaging methods for the study of intact tissues and cell structures [276-284]. Similar to multiphoton fluorimetry, the SHG method has a few important advantages compared to the methods of linear spectroscopy and imaging, since the exciting near infrared light is weakly scattered by the tissue, while the second harmonic generation occurs already inside the tissue, in the small focal region of the focused laser beam. This provides high spatial resolution, acceptable probing depth, and decoupling of exciting and detected signals. Strongly focused laser beams with high power density and very short pulse duration of tens and hundreds of femtoseconds allow the harmonic generation in a living tissue practically without any damage, because of small interaction time and the total energy, too small for molecular ionisation [284].

SHG is a nonlinear optical process of the second order that can occur only in the media, possessing no central symmetry. The method can be used for imaging highly-ordered structure proteins without any exogenous labels, as well as for probing biological membranes with high specificity to the membrane type [277]. In turn, the third harmonic generation is very sensitive to the refractive index variations and can be used to visualise interphase regions in cells and tissues [285].

Due to its coherent nature, the wave of the second harmonic is mainly radiated in the forward direction. For thicker and strongly scattering samples a part of the SHG signal is scattered. The major part of the SHG radiation still propagates in the forward direction. A great part of structural information encoded in the forward signal is lost in the backward signal due to multiple scattering events. Nevertheless, the mode of SHG backscattering allows the study of upper layers of intact skin or whole animals that could not be implemented in the transmission mode [24, 278, 284, 286].

The SHG in skin is provided, mainly, by the dermis due to its major component, the collagen that possesses considerable nonlinear susceptibility [280-283]. Obviously, the reduction of scattering due to optical clearing for the incident long-wave light and, particularly, for the short-wave second-harmonic detected light can improve the SG images of the collagen structures of the dermis in the forward direction and worsen the signal in the reflection mode.

Wen et al. [76] injected glycerol solutions into the rat skin *in vivo*, and after 10 minutes the samples were

cut for SHG imaging. With the solution having the concentration 75% the dehydration (diameter reduction) of dermis collagen fibres was observed.

Fig. 10 illustrates the change of packing density of the dermis collagen fibres under its interaction with 50% PEG-400 solution. The images were obtained using the SHG microscopy.

Fig. 10 The packing density change of collagen fibres in the rat skin *ex vivo* under the action of 50% aqueous solution of PEG-400: the initial state (a); immediately after the OCA application (b), and in 15 minutes after the application of OCA (c). The images were obtained by means of SHG microscopy. The incident light wavelength was 790 nm, the detected wavelength 395 nm.

Yeh et al. [121] and Wen et al. [76] demonstrated that for the collagen-based connective tissues (samples of tendon and model tissues) the optical clearing process using the high-concentration glycerol (13 M) manifested itself in considerable reduction of the backscattered signal intensity and the increase of the transmitted signal.

For deeper probing of the murine skeletal muscle and tendon samples using three-dimensional SHG

microscopy in the transmission mode the authors of Ref. [24] used the 50% solution of glycerol. They obtained the increase of SHG imaging depth by 2.5 times in the muscle tissue. The amplification of the signal was also due to better packing of the fibrils. It was also shown that the axial attenuation of the direct SHG signal is reduced with the increase of the glycerol concentration (25, 50, and 75%) [72]. The backward SHG signal considerably decreased in the process of optical clearing due to the scattering reduction and the change of the local density of dipoles, producing the second harmonic [72].

The Raman nonlinear spectroscopy also finds its application in studying the mechanisms of optical clearing. Thus, e.g., in Ref [287] the signal of coherent anti-Stokes Raman scattering (CARS) served as the reference in the study of DMSO impact on the collagen structure and, therefore, the loss of SHG signal and the reduction of light scattering by human skin. Hirshburg et al. [110] found the correlation between the rate of the water loss by the rodent skin *ex vivo* under the effect of different OCAs and the potential of optical clearing, which was determined by the slope of the line μ'_s (before clearing)/ μ'_s (after clearing).

Raman spectroscopy is a potential noninvasive method for studying the bone development and for diagnostics of bone diseases [288]. Essential amplification of commonly undetectable Raman signals from internal tissues can be implemented by means of optical clearing of near-surface layers of the tissue that shade the object of study [288, 289]. In this way the transcutaneous spectroscopy of the rat tibial bone *in vivo* was performed under the skin optical clearing with glycerol [288]. The process of porcine skin optical clearing was studied *in vitro* by means of Raman microspectroscopy [289]. The intensity of Raman peaks at the depth of 400 μm was increased by 2-4 times. Besides, the shifts of the peaks in the Raman spectrum of skin at different concentrations of glycerol were observed [289].

The confocal Raman spectroscopy can also be used for *in vivo* monitoring of penetration of clearing agents and permeability enhancers (particularly, DMSO) into the skin *stratum corneum* [290].

5.6 Terahertz spectroscopy

The terahertz (THz) frequency range is intermediate between the IR and microwave frequency ranges (the frequency $\nu = 1$ THz corresponds to the free-space wavelength $\lambda = 300 \mu\text{m}$). Recent progress in generating and detecting the THz radiation and its usage offer its promising applications in biology and medicine [291]. Since the energy of THz photons is too small to ionise molecules in biological systems, the THz spectroscopy is expected to be a promising method of study in biology and medicine. Many vibrational transitions of biomolecules lie just in this frequency range, and the light scattering is not so strong as in the visible and near infrared spectral region, so that the excitation by ultrashort pulses can allow the study of a wide

frequency range in a single measurement with high temporal resolution [3, 292-294]. Terahertz spectroscopy allows the determination of the medium complex refractive index in a single measurement, which is important for creating a functional THz tomograph with high sensitivity to the change of metabolite concentrations and precise mapping of the pathologic process boundaries [3]. At the same time, due to the difficulties associated with the specific features of such objects, the biomedical applications are considered as medium-term and long-term goals of THz studies [291].

Keeping in mind that the frequencies of the THz range mainly coincide with the frequencies of rotational and vibrational transitions of the organic molecules, the study of this range may be used for spectral identification of molecules [295]. This circumstance allows the use of THz technologies in addition to the spectroscopic techniques, based on using electromagnetic waves of other frequency ranges. In this connection the development of spectroscopic methods of studying biological tissues in the THz region, providing the detection and visualisation of metabolic and pathologic processes, attracts great interest in recent years, especially as an additional channel of getting information in multimodal systems, in combination with optical methods [296, 297]. In such applications the immersion clearing of the tissue solves many problems of applying optical methods at the expense of reversible reduction of light scattering, and the accompanying dehydration of the tissue facilitates greater permeability for THz radiation due to the reduction, also reversible, of absorption by water contained in the tissue. Direct *in vitro* measurements of the effect of porcine muscle tissue dehydration under the immersion of the sample in glycerol during 30 minutes have shown essential reduction of THz radiation absorption in the region 0.1–1.5 THz, measured using the method of frustrated total internal reflection [292]. The loss of the sample mass amounted to 10% of its initial mass, and the transmission in the visible wavelength range increased upon the average by 5%. This experiment also demonstrated the high sensitivity of the THz range to the concentration of water in tissues, which apparently can serve as one of the criteria for pathology recognition [296].

The influence of dehydration by freezing (lyophilisation) of tissue samples of different rat organs (kidney, diaphragm, liver, rectum, stomach) on the THz spectra in the range 0.4–2.2 THz was studied in Ref. [294]. Among the drawbacks of this method it is worth mentioning the impossibility of *in vivo* studies. Moreover, deep and long freezing of the tissue leads to essential changes of its structure. The dehydration of a tissue can be also implemented by the thermal impact (e.g., using a fan), i.e., by mere drying [298]. However, this method suffers from the same drawbacks as the one presented above.

Obviously, the implementation of *in vivo* dehydration requires temporary and reversible changes

in the tissues under study. In Ref. [299] it is proposed to perform the dehydration of muscle tissue using the hyperosmotic agents. As clearing agents the authors used PEG-600, dehydrated glycerol, and propylene glycol. The possibility of reducing the absorption of radiation in the range 0.25 – 1.6 THz under its propagation through the tissue by applying OCA has been unambiguously proved, which offers a possibility of essential increase of the probing depth of a tissue with such radiation. Depending on the frequency of radiation within the studied range, the absorption coefficient reduction approached 60%, and the necessary application time was 8 – 10 minutes, which is acceptable for using this technology in medical practice [299].

5.7 Determination of diffusion coefficients for clearing agents and drugs in tissues

In spite of multiple studies related to the control of optical properties of tissues, the problem of determining diffusion coefficients for hyperosmotic fluids in tissues still remains insufficiently studied. Due to the complex multicomponent structure of tissues and nonlinear character of diffusion processes the measurement of diffusion coefficient of hyperosmotic fluids in tissues is a rather difficult scientific problem. According to the present-day conceptions, the diffusion of different substances in tissues occurs in several stages. At the first stage the penetration of diffusing agents into the intercellular and interfibrillar space and their interaction with cell membranes and interstitial matrix of tissues occurs. At the next stage the transmembrane diffusion of the agent into cells probably occurs, accompanied by the change of the intracellular osmotic pressure. One should not exclude also the possibility of transformation of the membranes themselves, related to the change of the intercellular fluid properties. Under the sufficient saturation of interfibrillar space of a connective tissue with the clearing agent its interaction with the tissue fibrils will obviously take place.

However, the use of sufficiently simple optical and diffusion models allows the solution of the present problem without significant loss of accuracy, and the natural dispersion of optical and structural properties of tissues makes this simplification acceptable. The resulting values of diffusion coefficients can be successfully used in mathematical models, describing the processes of interaction between the hyperosmotic immersion fluids and the tissues, which, in particular is rather urgent in the study of processes, related to transcutaneous delivery of medicinal preparations and metabolic agents.

At present a number of biophysical methods and techniques for determining the diffusion coefficients of different agents exist [300-313], but, unfortunately, only a small part of the methods can be successfully applied to assess the diffusion coefficients in tissues. The available methods are mainly based on using the fluorescence measurement techniques or radioactive

Table 1 Light penetration depth increase by optical clearing agents in different diagnostic methods.

Diagnostic method	Tissue	OCA, refractive index	Spectral range	Light penetration depth increase	Reference
Confocal microscopy	Dehydrated murine adipose tissue, muscle tissue, myocardium, brain	Murray's Clear, 1.55	334 – 799.3 nm	~35 folds	[250]
Two-photon microscopy	Human skin <i>ex vivo</i>	Dehydrated glycerol, 1.47	Excitation – 700-1000 nm, detection – 370-670 nm	2 folds	[23]
Confocal/two-photon microscopy	Cellulose membrane	FocusClear, 1.46	Excitation – 820 nm, detection – 390-465 nm	2 folds	[251]
3-D second-harmonic microscopy (direct wave mode)	Muscle tissue of a mouse	50% glycerol solution, 1.395	Excitation – 890 nm, detection – 445 nm	2.5 folds	[24]
Raman microspectroscopy	Porcine skin <i>in vitro</i>	80% glycerol solution, 1.46	785 nm	4 folds	[289]
THz spectroscopy	Bovine muscle tissue	Dehydrated glycerol, 1.47	0.25-1.6 THz	45%	[299]
	Mouse embryo	50% glycerol solution, 1.395	1310 nm	4.5 folds	[59]
OCT	Human skin <i>in vivo</i>	Pre-polymer mixture on the base of propylene glycol and polyethylene glycol, 1.47	1305 nm	1.2 folds	[70]
	Whole blood, diluted by two times with saline	6.5% glycerol solution, 1.34	820 nm	2.4 folds	[128]

labels for recording the flow of diffusing substance. The use of fluorescence measurement technique is impossible for the assessment of diffusion coefficients of non-fluorescent substances (e.g., aqueous solutions of glucose or glycerol), and the use of radioactive substances can be undesirable in the case of *in vivo* measurements. Moreover, the high sensitivity of fluorescence and radioactive measurements makes their use difficult also because of the natural metabolic activity of the organism, which introduces additional errors into the measurement process. The method that allows the assessment of diffusion coefficients of non-fluorescent hyperosmotic fluids in tissues was proposed in 1997 [43] and later developed in Refs. [47, 90]. This method is based on measuring the time dependence of the tissue scattering characteristics under the action of hyperosmotic fluids and can be successfully used for both *in vitro* and *in vivo* measurements.

The process of immersion fluid transport in fibrous tissues can be described using the free diffusion model. In this case the following assumptions related to the transport process are commonly made: 1) only the concentration diffusion takes place, i.e., the exchange flux of the immersion fluid or drug into the tissue and of

water from the tissue at a given point is proportional to the gradient of concentration of the clearing agent at this point; 2) the diffusion coefficient is constant at all points in the studied tissue sample.

Geometrically the tissue sample is presented as a plane-parallel plate of final thickness. Since the area of the upper and lower surfaces of this plate is much larger than the side area, it is possible to neglect the edge effects and solve the one-dimensional diffusion problem described by the equation

$$\frac{\partial C(x,t)}{\partial t} = D \frac{\partial^2 C(x,t)}{\partial x^2}, \quad (4)$$

presenting the second Fick law, where $C(x,t)$ is the concentration of the immersion agent (e.g., glucose) in the skin (g/ml); D is the diffusion coefficient (cm^2/s); t is the time, during which the diffusion process occurs (s); x is the spatial coordinate along the sample thickness direction (cm). Since in the experiments the volume of the immersion fluid, as a rule, significantly exceeds the volume of the tissue sample, the appropriate boundary conditions have the form

$$C(0, t) = C_0 \quad \text{and} \quad \frac{\partial C(l, t)}{\partial x} = 0, \quad (5)$$

where C_0 is the glucose concentration in the solution (f/ml); l is the tissue sample thickness (cm). The second boundary condition reflects the fact that the diffusion of the immersion fluid into the skin sample occurs only from one side of the sample, i.e., from the dermis side. In the case, when the immersion agent diffuses from both sides of the tissue sample (such situation arises, e.g., in experiments with sclera or dura mater in vitro), the boundary conditions have the form:

$$C(0, t) = C_0 \quad \text{and} \quad C(l, t) = C_0. \quad (5^*)$$

The initial conditions express the absence of the immersion agent in all internal points of the sample before its incubation into the solution, i.e.,

$$C(x, 0) = 0. \quad (6)$$

The solution of the diffusion equation (4) with the initial (6) and boundary (5) conditions taken into account has the form:

$$C(x, t) = C_0 \left(1 - \sum_{i=0}^{\infty} \frac{4}{\pi(2i+1)} \sin\left(\frac{(2i+1)\pi x}{2l}\right) \times \exp\left(-\frac{(2i+1)^2 D \pi^2 t}{4l^2}\right) \right).$$

The mean concentration of the immersion solution in the tissue sample $C(t)$ as a function of time is determined by the expression

$$C(t) = C_0 \left(1 - \frac{8}{\pi^2} \sum_{i=0}^{\infty} \frac{1}{(2i+1)^2} \times \exp\left(-\frac{(2i+1)^2 t \pi^2 D}{4l^2}\right) \right). \quad (7)$$

In the case of double-side diffusion the solution of the diffusion equation (4) with the initial (6) and boundary (5*) conditions has the form

$$C(x, t) = C_0 \left(1 - \sum_{i=0}^{\infty} \frac{4}{\pi(2i+1)} \sin\left(\frac{(2i+1)\pi x}{l}\right) \times \exp\left(-\frac{(2i+1)^2 D \pi^2 t}{l^2}\right) \right).$$

In this case the mean concentration of the immersion solution in the tissue sample $C(t)$ as a function of time

is determined by the expression

$$C(t) = C_0 \left(1 - \frac{8}{\pi^2} \sum_{i=0}^{\infty} \frac{1}{(2i+1)^2} \times \exp\left(-\frac{(2i+1)^2 t \pi^2 D}{l^2}\right) \right). \quad (7^*)$$

In the first approximation Eq. (7) can be rewritten as

$$C(t) \approx C_0 \left(1 - \exp\left(-t \pi^2 D / l^2\right) \right), \quad (8)$$

and Eq. (7*) as

$$C(t) \approx C_0 \left(1 - \exp\left(-t \pi^2 D / l^2\right) \right). \quad (8^*)$$

Note, that Eqs. (8) and (8*) correspond to the equation describing the diffusion through a thin penetrable membrane.

As the immersion agent penetrates into the tissue, the increase of the refractive index of the interstitial fluid will be observed. The assessment of the refractive index of the interstitial fluid depending on the diffusion time can be performed basing on the Gladstone-Dale law, according to which the refractive index of a mixture of non-interacting liquids is a sum of the refractive indices of individual components multiplied by the volume fractions of these components [314]. Mathematically the Gladstone-Dale law is written as

$$n_{\Sigma} = \sum_i n_i C_i, \quad \text{where} \quad \sum_i C_i = 1. \quad (9)$$

Here n_{Σ} is the refractive index of the multicomponent mixture of non-interacting liquids; n_i and C_i are the refractive indices and volume fractions of each component. In the case of two-component solutions (in our case the components are the interstitial fluid and the solution of the immersion agent) the Gladstone-Dale law has the form

$$n_i(t) = (1 - C(t)) n_{base} + C(t) n_{osm}, \quad (10)$$

where n_{base} is the refractive index of the interstitial fluid at the initial moment of time, and n_{osm} is the refractive index of the hyperosmotic immersion fluid (e.g., glucose or glycerol solution). For the aqueous solution of glucose the value of the refractive index (n_{gl}) can be found from the expression [315]

$$n_{gl}(\lambda) = n_{H_2O}(\lambda) + 0.1515 \times C_{gl}, \quad (11)$$

where $n_{H_2O}(\lambda)$ is the spectrally dependent refractive index of water ($[\lambda] = \text{nm}$), and C_{gl} is the glucose concentration in the solution (g/ml). The spectral dependence of the water refractive index has the form [316]:

$$n_{H_2O}(\lambda) = 1.3199 + \frac{6.878 \times 10^3}{\lambda^2} - \frac{1.132 \times 10^9}{\lambda^4} + \frac{1.11 \times 10^{14}}{\lambda^6}. \quad (12)$$

The optical characteristics of skin are determined by the optical properties of dermis (since its thickness is dominating with respect to the thickness of other skin layers). Hence, the optical model of the tissue can be presented as a layer having the thickness l , containing the scatterers in the form of infinitely long thin dielectric cylinders, parallel to the sample surface. In the first approximation one can assume that in the process of interaction of the tissue with the immersion fluid the scatterer size does not change. For densely packed tissues, i.e., the tissues with sufficiently large volume fraction of scatterers, to which the skin belongs, it is important to allow for the interference effects that arise because each scatterer is located in the near-field zone of radiation, scattered by the other scatterers, described by introducing the packing factor [3, 317]. For a system of thin dielectric cylinders, arranged parallel to each other, the packing factor has the form $(1-\varphi)^3/(1+\varphi)$ [317], where φ is the volume fraction of the scatterers. Thus, the expression describing the tissue scattering coefficient can be written in the form [90]:

$$\mu_s = \frac{\varphi \pi x^3}{a} \frac{(m^2 - 1)^2}{8} \left(1 + \frac{2}{(m^2 + 1)^2} \right) \frac{(1-\varphi)^3}{1+\varphi}. \quad (13)$$

The time dependence of the collimated transmission coefficient of the tissue sample immersed in the solution of a hyperosmotic liquid or medicinal preparation has the form:

$$T_c(t) \approx \exp\left(-(\mu_a + \mu_s(t)) \times l\right), \quad (14)$$

where μ_a is the absorption coefficient of the tissue sample.

Equations (4)-(13) determine the dependence of the collimated transmission coefficient upon the concentration of the immersion fluid solution (e.g., the solution of glucose or glycerol) in the sample of the tissue, i.e., formulate the direct problem. The inverse problem is to reconstruct the values of the diffusion coefficient from the time dependence of the collimated transmission. This problem is solved by minimisation of the target functional

$$f(D) = \sum_{i=1}^{N_t} \left(T_c(D, t_i) - T_c^*(t_i) \right)^2, \quad (15)$$

where N_t is the total number of experimental points, obtained by recording the time dependence of collimated transmission at the fixed wavelength: $T_c(D, t)$ is the value of the transmission coefficient, calculated using the formula (14) at the time moment t for the given values of D ; $T_c^*(t)$ is the experimentally measured value of the transmission coefficient at the time moment t .

The flowchart of the program implementing the present algorithm is shown in Fig. 11. The main steps of the algorithm include:

1) Specification of the initial parameters for the chosen wavelength (the absorption coefficient μ_a , the refractive index of the interstitial fluid n_s , the refractive index of the glucose solution or a drug n_b , the thickness of the tissue sample l) obtained either from independent measurements, or from literature data.

2) Specification of the initial value of the diffusion coefficient D . The initial value of D can be obtained from the analysis of variation of the sample collimated transmission basing on the equation

$$\tau = - \frac{\sum_{j=1}^n t_j^2}{\sum_{j=1}^n (t_j \ln y_j)},$$

where t_j is the time moment of measuring each value of T_c , T_c is the collimated transmission coefficient, $y = 1 - T_c/A$; A is the maximal value of the collimated transmission coefficient; τ is the diffusion time constant

(s); $\tau = \frac{l^2}{\pi D}$ in the case of single-side diffusion and

$\tau = \frac{l^2}{\pi^2 D}$ in the case of double-side diffusion.

3) Calculation of the kinetics of collimated transmission versus time for given D and t_j using the Bouguer's law (Eq. (14)).

4) Comparison of the calculated and measured values of collimated transmission coefficients. If the difference does not exceed the prescribed error, the value of D is found and the process is terminated, otherwise the value of D is changed using the minimisation simplex method [318], and the procedure is repeated till the prescribed accuracy is achieved.

As an example, Table 2 presents the values of diffusion coefficients of glucose aqueous solutions in tissues, measured using the above method at the room temperature (about 20°C). However, for medical applications the most interesting are the diffusion coefficient values measured at the physiological temperature (nearly 37°C). Since the direct measurement of the diffusion coefficients at the physiological temperature requires thermostating of the

Table 2 Glucose diffusion coefficients in biotissues [47].

Tissue	Immersion fluid	Diffusion coefficient, $\times 10^{-6}$, cm^2/s , measured at 20°C	Diffusion coefficient, $\times 10^{-6}$, cm^2/s , at the temperature 37°C
Eye sclera	Aqueous solution of glucose, 0.2 g/ml	0.57 ± 0.09	0.91 ± 0.09
Eye sclera	Aqueous solution of glucose, 0.3 g/ml	1.47 ± 0.36	2.34 ± 0.36
Eye sclera	Aqueous solution of glucose, 0.4 g/ml	1.52 ± 0.05	2.42 ± 0.05
Pachymeninx	Aqueous solution of glucose, 0.2 g/ml	1.63 ± 0.29	2.59 ± 0.29
Skin	Aqueous solution of glucose, 0.4 g/ml	1.10 ± 0.16	1.75 ± 0.16
Skin (<i>in vivo</i>)	Aqueous solution of glucose, 0.4 g/ml		2.56 ± 0.13

Fig. 11 Flowchart of the program for calculating the diffusion coefficients of glucose and drugs basing on the kinetics of collimated transmission of tissue samples.

tissue sample that essentially complicates the experimental setup and measurement technique, one can determine the diffusion coefficient at the physiological temperature theoretically, using the following dependence of the diffusion coefficient upon the temperature [319]:

$$D(T_2) = D(T_1) \frac{T_2}{T_1} \frac{\eta(T_1)}{\eta(T_2)},$$

where $\eta(T)$ is the viscosity of the medium where the diffusion occurs (e.g., water) at the given temperature T . In our case $T_1 = 20^\circ\text{C}$ and $T_2 = 37^\circ\text{C}$. In Table 2 (4-th column) the values of the diffusion coefficients calculated for the temperature 37°C are presented.

From Table 2 it is well seen that the temperature increase from room to physiological, i.e., by 17°C , causes the increase of the diffusion coefficient nearly by 1.5 times.

6 Conclusion

A review of specific features and methods of optical clearing and related interaction of light with biological tissues is presented. The impact of the OCA on a tissue allows efficient control of the optical properties, particularly, the reduction of tissue scattering coefficient, which facilitates the efficiency increase in different methods of optical imaging (optical biopsy) in medical applications. Both immersion and compression techniques of optical clearing possess considerable potential for many diagnostic, therapeutic, and surgical methods, in which the laser impact on the target site hidden in the tissue is used.

The work was supported by Russian Presidential grant NSh-703.2014.2, the Government of the Russian Federation grant 14.Z50.31.0004 (EAG, ANB, IYuY, and VVT), RFBR grant 14-02-00526 (VVT), and The Tomsk State University Academic D.I. Mendeleev Fund Program (EAG, ANB, YuPS, and VVT).

The authors wish to thank Prof. Dan Zhu (HUST, China) and her PhD students for their help in the obtaining of the SHG-images.