For the last 20 years, the optical method as a tool for clinical functional imaging of physiological conditions, cancer diagnostics and therapies has been of great interest owing to its unique informative features, simplicity, safety and low cost in contrast to conventional x-ray computed tomography, MRI and ultrasound imaging [1–3]. However, the main limitations of the optical imaging techniques, including optical diffusion tomography, optical coherence tomography, confocal and nonlinear microscopy, reflectance spectroscopy and others, are due to strong light scattering in tissue layers and blood, which cause low contrast and spatial resolution, as well as a small probing depth [3–5].

One of the prospective solutions of the problem is a reduction of the light scattering due to the tissue layers and blood, which provides improvement of image quality and precision of spectroscopic information from tissue depth [6,7]. In laser surgery, the reduction of light scattering by a tissue results in a decrease of irradiating light beam distortion, and the possibility of its sharp focusing, as well as reducing the radiant exposures [8–10]. Various physical and chemical actions, such as compression [11], stretching [12], dehydration [13], coagulation [14] and impregnation by biocompatible chemical agents [6–10], are widely described in the literature as tools for controlling tissue optical properties.

In this article, we discuss the optical immersion method based on refractive index matching of scatterers (e.g., collagen, elastin fibers, cells and cell compartments) and the ground material (interstitial fluid and/or cytoplasm) of tissue and blood under the action of exogenous optical clearing agents. We analyze the optical clearing of fibrous and cell-structured tissues and blood from the point of view of receiving more valuable, normally hidden, information from spectroscopic and polarization measurements, confocal microscopy, optical coherence and optical projection tomography, as well as from nonlinear spectroscopies, such as two-photon fluorescence and second-harmonic generation techniques. Some important applications of the immersion technique to glucose sensing, drug delivery monitoring, improvements of image contrast and imaging depth, nondistortive delivery of laser radiation and precision tissue laser photodisruption, among others, are also described.

**Keywords:** blood • laser therapy • optical clearing agents • optical diagnostics • optical immersion • tissues

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**Main mechanisms of tissue optical immersion clearing**

In general, the scattering coefficient ($\mu_s$) and scattering anisotropy factor ($g$) of a tissue is dependent on refractive index mismatch between cellular tissue components: cell membrane; cytoplasm; cell nucleus; cell organelles; melanin...
granules; and the extracellular fluid. For fibrous (connective) tissue (eye scleral and corneal stroma, skin dermis, cerebral membrane, muscle, vessel wall noncellular matrix, female breast fibrous component, cartilage, tendon, etc.), index mismatch of interstitial medium and long strands of scleroprotein (collagen-, elastin-, or reticulin-forming fibers) is important [15].

The nucleus and the cytoplasmic organelles in mammalian cells that contain similar concentrations of proteins and nucleic acids, have refractive indices that fall within a relatively narrow range (1.38–1.41) at a wavelength of 589 nm [16]. The measured refractive index for the nuclei is \( n_w = 1.39 \) [17]. The ground matter index is usually taken as \( n_0 = 1.35–1.37 \) [3]. The scattering particles themselves (organelles, protein fibrils, membranes and protein globules) exhibit a higher density of proteins and lipids in comparison with the ground substance and, thus, a greater index of refraction (\( n_1 = 1.39–1.47 \)) [18]. The refractive index of the connective-tissue fibers is approximately 1.41 and depends on the hydration of collagen, its main component [18]. The refractive index of the interstitial liquid, as well as human blood plasma, is approximately 1.33–1.35, depending on the wavelength [3,19]. The main scatterers in blood are red blood cells (RBCs). RBCs are cells without nuclei; they contain approximately 70% water, 25% hemoglobin, and 5% lipids, sugars, salts, enzymes and proteins [20]. The refractive index of dry RBCs at a wavelength of 550 nm falls within a range of 1.61–1.66 [21]. A hemoglobin (Hb) concentration of 32 g/dl represents a typical Hb concentration and the index of the solution is approximately 1.42 [22]. For human whole blood, depending on the wavelength, the index is approximately 1.36–1.40 [3].

The optical immersion technique is based on the impregnation of a tissue by a bio-compatible chemical agent, which may have some hyperosmotic properties. The OCAs frequently used are glucose, dextrose, fructose, glycerol, mannitol, sorbitol, propylene glycol, polyethylene glycol, 1,3-butanediol, 1,4-butanediol and their combinations, x-ray contrasting agents (verografin, trazograph, hypaque, etc.) [6–10,15,23–33].

There are a few main mechanisms of light scattering reduction induced by an OCA [13,23–30]: dehydration of tissue constituents; partial replacement of the interstitial fluid by the immersion substance; and structural modification or dissociation of collagen.

The first mechanism is characteristic only for highly hyperosmotic agents [13,29]. For fibrous tissue similar to sclera, dura mater, demiris, and so on, the second mechanism is prevalent for all tested chemical agents because their molecule sizes are much less than the mean cross-section of interfibrillar space [3,23–29]. Both the first and the second processes mostly cause matching of the refractive indices of the tissue scatterers (cell compartments, collagen and elastin fibers) and the cytoplasm and/or interstitial fluid.

The refractive index matching is manifested in the reduction of the scattering coefficient (\( \mu_s = 0 \)) and increase of single scattering directness (\( g = 1 \)). For fibrous tissues such as skin dermis, eye sclera, dura mater, tendon, and so on, \( \mu_s \) reduction can be very high [6,7,23,24,28,31,32].

Structural modification can lead to tissue shrinkage — that is, to the near-order spatial correlation of scatterers and, as a result, the increased constructive interference of the elementary scattered fields in the forward direction and destructive interference in the perpendicular direction of the incident light that may significantly increase tissue transmittance even at some refractive index mismatch [7]. For some tissues and for the nonoptimized pH of clearing agents, tissue swelling may take place that could be considered as a competitive process in providing tissue optical clearing [7,26,33,34].

The optical clearing process in collagen-based tissues may involve a change in the supramolecular structure. Collagen reversibly solubility in sugars and sugar alcohols may take place [30]. Agent-induced destabilization of collagen structures may lead to an additional reduction of optical scattering in tissue owing to less size of the main scatterers [30].

For the use of hyperosmotic agents, osmotic pressure may play a significant role. On one hand, the osmotic pressure causes the generation of fluid flows and controls intensities of these flows;
however, on the other hand, rather strong osmotic pressure may destroy tissue structure [35]. This is a major physicochemical mechanism of OCA toxicity.

For propagation of polarized light in fibrous tissue, it was shown that at a reduction of scattering, the degree of linearly polarized light improves [15]. As far as the tissue is immersed, the number of scattering events decreases and the residual polarization degree of transmitted linearly polarized light increases. As a result, the kinetics of the average transmittance and degree of polarization of the tissue are correlated. OCA-induced optical clearing leads to an increase in the length of depolarization of a number of tissues [15].

Figure 1 illustrates the change of the reduced scattering coefficient, \( \mu'_s = \mu_s (1 - g) \) of both tissue samples taken from rat skin human dura mater and measured at wavelength 589 nm under action of anhydrous glycerol (\( n = 1.47 \)), and aqueous mannitol solution (0.16 g/ml) (\( n = 1.357 \)), respectively [24,32]. It is seen that the administration of OCAs into the tissues changes their scattering properties. In the course of clearing, the reduced scattering coefficient of dura mater decreases approximately 40%, and that of skin decreases 75% in approximately 20 min. Differences in the degree of tissue clearing can be explained by differences in refractive indices of the used OCAs, their osmolarity and initial state of turbidity (tissue structure). The swelling of dura mater samples was observed [32], whereas skin shrank during the clearing [24].

Not only soft but also hard tissues could be effectively cleared, which opens the way for the development of the least-invasive techniques for laser diagnostics and therapy of brain and other soft tissue hidden under bone, cartilage or tendons. For example, optical immersion clearing of the cranial bone under action of anhydrous glycerol was investigated [31]. It was shown that exposure of a cranial bone sample in an hour causes the decreasing of reduced scattering coefficient of superficial tissue layers by approximately 25% at the wavelength range of 1400–2000 nm. In this case, the main role in the clearing process is the replacement of water in the interstitial space by the immersion substance, owing to the cranial bone-specific structure having a rather high porosity [31].

Blood immerses or goes through practically all tissues, and its scattering properties are very strong and anisotropic; thus, its clearing is of great importance for the optical technologies successful application. The refractive-index mismatch between erythrocyte cytoplasm and blood plasma, as well as specific size and structure, cause the scattering properties of blood [3]. The refractive index of erythrocyte cytoplasm is defined mostly by Hb concentration [36]. The volume and shape of a single erythrocyte are defined by blood plasma osmolarity [36]. Blood scattering also depends on aggregation or disaggregation capability of RBCs [37].

Upon introduction of OCAs into blood, the refractive index of the blood plasma increases and becomes comparable with that of RBCs. For example, in whole blood diluted to twice of its volume by saline with the addition of 6.5% glycerol, the total attenuation coefficient was reduced from 4.2 to 2.0 mm⁻¹, and the optical penetration at 820 nm was correspondingly increased to 117%. For the other agents tested (glucose, dextrans, propylene glycol and trazograph), the enhancement of penetration was 20–150% [7,38]. It was shown that minimal light scattering occurs at a glucose concentration in blood of 0.65 g/ml. In this case blood is totally immersed. However, residual scattering remains due to differences in spectral dependencies of refractive indices of glucose solution and RBCs [39]. Evidently, such large concentrations of glucose could only be applied locally and for a short time to avoid damage to blood cells and vessel wall tissue. However, using endoscopic optical imaging techniques (optical coherence tomography [OCT] or confocal microscopy [CM]) and controllable injection of small amounts of glucose in the vessel lumen close to imaging area may significantly help to image an atherosclerotic plaque through a blood layer with a high contrast.

There is also the possibility of applying an immersion agent, a small amount of hemoglobin, which could be released owing to local hemolysis of RBCs within the vessel area close to endoscopic optical probe [40]. A 30–40% reduction of the scattering coefficient of blood in the spectral, ranging from 400 to 1000 nm, with an increase in the local hemolysis (up to 20% of RBC in the vicinity of optical probe) was demonstrated theoretically [40].

Figure 2. Time-dependent reduced scattering spectra of rabbit eye sclera in vivo and human sclera in vitro calculated at a wavelength of 700 nm after administration of 40% glucose solution. Symbols and solid curves correspond to experimental data and the result of their approximation, respectively. The values are obtained from data presented in [8,41].
It was also found that optical clearing of blood is defined not only by refractive-index matching, but also by changes in the size of RBCs and in their aggregation ability when chemicals are added [38].

For hematous tissue, such as the liver, its impregnation by solutes with different osmolarity also leads to refractive index matching and reduction of scattering coefficient, but the effect is not so pronounced as for fibrous tissues which change size as a result of osmotic stress [7].

The results presented above relate to in vitro investigations. It is known that at in vivo application of the designed optical immersion technology, additional factors such as metabolic reaction of living tissue on clearing agent application, the specificity of tissue functioning and its physiological temperature can significantly change kinetic characteristics and the magnitude of the clearing effect [8,9,27,28,33]. In a living tissue, the relative refractive index is a function of tissue physiological or pathological state. Depending on the specificity of the tissue state, the refractive index of the scatterers and/or the background may be changed (increased or decreased), and therefore light scattering may correspondingly increase or decrease [3,7].

For example, Figure 2 presents the kinetics of reduced scattering coefficients of rabbit eye sclera in vivo and human sclera in vitro calculated at a wavelength of 700 nm on the basis of experimental data at different time intervals after administration of 40% glucose solution [8,41]. The clearing degree of scleral tissue in vivo is approximately 1.7 fold — that is, less than the clearing degree of sclera in vitro (~2.7 fold). In vivo conditions, the clearing agent was washed out from the area of its impregnation (simple dropping of glucose into the eye was used), and induced a reaction of physiological processes of the living tissue, and thus the clearing efficiency is somewhat less.

Injection of OCA as glycerol and glucose into skin also influences the state of blood microcirculation in dermis. The OCA penetrates vessel walls, interacts with blood cells and leads to local dehydration of tissue and cells [42]. It was shown on chick chorioallantoic membrane that the effects depend on the dosage of OCAs, including volume and concentration, and decrease with the dosage of agents [43].

Enhancers of OCA diffusion
Ethanol, propylene glycol, dimethylsulfoxide (DMSO), linoleic and oleic acids, azone and thiazole are typically used as enhancers of OCA diffusivity through stratum corneum (SC) [44–50]. Sometimes, DMSO alone is used as the OCA owing to its own extremely high permeability (as a polar aprotic solvent of SC lipids) and its high index of refraction [48].

Ethanol is a solvent known to modify the skin barrier property. At high concentrations (~40%), ethanol greatly enhances pore transport, owing to bigger pores and/or pore density of the epidermal membrane at its action [49,51].

The solvation of keratin within the SC by competition with water for the hydrogen bond binding sites, and the intercalation in the polar headgroups of the lipid bilayers by propylene glycol, are postulated as mechanisms for the penetration-enhancing effects of propylene glycol [50]. It was shown that mixtures of different OCAs with propylene glycol increase the effectiveness of the optical clearing effect, and that the clearing effect induced by propylene glycol itself is worse than that induced by the OCAs [47].

For thiazole and azone, a possible mechanism would be the increase of fluidity of the hydrophobic SC regions and the corresponding reduction of the permeation resistance of the horny layer against drug substances [46,47].

As distinct from chemical enhancers, physical methods for transdermal agent delivery have two features: interaction between the enhancer and the agents being delivered is absent; and they reduce the risk of additional skin irritation [52]. To reduce the barrier function of skin epidermis, physical methods such as tape stripping [53], microdermabrasion [54], low intensive and high intensive laser irradiation of skin surface [55,56], iontophoresis [57], ultrasound [58] and photomechanical (shock) waves [52], needle-free injection [59], photothermal and mechanical microperforation [60,61], or microdamaging of epidermis [62] were proposed.

Applications of tissue optical clearing to different diagnostic & therapeutic techniques
Over the last 10 years, noninvasive or minimally invasive spectroscopic techniques have achieved a widespread occurrence in biomedical diagnostics, for example, OCT [3,63–65], visible and near-infra-red (NIR) spectroscopy [3,27,66], fluorescent [67–69] and polarization spectroscopy [3,15,17,70], as well as others. Spectroscopic techniques are capable of providing a deep imaging of tissues that could provide information regarding blood oxygenation and detect cutaneous and breast tumors [69–71], whereas CM [1,72–75], OCT [3,63–65] and multiphoton excitation imaging [1–3] have been used to show cellular and subcellular details of superficial living tissues. Spectroscopic, OCT and photoacoustic techniques are applicable for blood glucose monitoring with diabetic patients [3,64,65,75–83].

Next, we discuss several diagnostic and therapeutic applications of the immersion technique.

Tissue imaging
Confocal microscopy
Confocal microscopy is widely used at present in various biomedical investigations for the visualization of the internal structure of biological tissues on a cellular and subcellular level [72,73]. A confocal microscope illuminates and detects the scattered or fluorescent light from the same small volume within the specimen. The main advantage of CM is its ability to optically section thick specimens [73]. This technique makes it possible to obtain high-quality (with a micrometer spatial resolution) images of cellular layers. A high image contrast and a high spatial resolution of CM are achieved through probing a small volume of the medium bounded by the size of the central focal spot, which is formed by a focusing optical system. The main limitation of CM in skin studies is high scattering that distorts the quality of cell images. The increase in the transparency of the upper skin layers can improve the penetration depth, image contrast and spatial resolution of CM.
Firstly, it was predicted theoretically on the basis of Monte Carlo simulation of the point-spread function of the confocal reflectance microscope that precise skin images are possible to obtain for much bigger depths, and reticular dermis can be investigated [74].

Recently, the experimental technique that allows for imaging of the microvasculature of various murine tissues by CM, to depths of up to 1500 µm below the specimen surface owing to optical clearing of thick tissue sections, was described [75]. Also, recently, the commercially available agent FocusClear™ has been suggested as an OCA for CM [84,85]. FocusClear is a non-toxic aqueous tissue-clearing solution, making biological tissues transparent with the minimal dehydration effect [201,301]. It was developed to obtain high-resolution images in fluorescent and nonfluorescent light microscopes without damaging slices and detailed morphology of tissues. FocusClear comprises of DMSO, diatrizoate acid, ethylenediaminetetraacetic acid, glucamine, β-nicotinamide adenine dinucleotide phosphate, sodium diatrizoate and a derivative of polyoxyalkalene (as an emulsifying agent and detergent) in a suitable aqueous solution [201]. The refractive index of the OCA is 1.46.

FocusClear has been successfully used for confocal visualization of insect brains (cockroach, Diploptera punctata) [84]. The application of the OCA has allowed 3D mapping at submicron resolution of an entire brain that measures more than 500 µm in thickness.

FocusClear was also applied to permeate and reduce the opacity of mouse colon and ileum [85]. Tissues were labeled with fluorescent probes and examined by CM with efficient fluorescence excitation and emission in the FocusClear solution. The method allowed for identification of spatiotemporal changes in crypt morphology of colon tissues from mice with DMSO sulfate sodium-induced colitis, as well as detection of transgenic fluorescent proteins expressed in the colon and ileum.

FocusClear could be a promising agent for in vivo tissue visualization, but it requires further investigations.

**Multiphoton microscopy**

Multiphoton excitation of molecules is a nonlinear process involving the absorption of two or more photons whose combined energy is sufficient to induce a molecular transition to an excited electronic state. The two-photon technique employs photons at the wavelength of the second harmonic of incident radiation,
coming exactly from the focal area of the excitation beam [2,3]. A unique advantage of multiphoton microscopy is the possibility to investigate 3D distributions of chromophores excited with ultraviolet radiation in thick samples. Such an investigation becomes possible because chromophores can be excited (e.g., at the wavelength of 350 nm) with laser radiation whose wavelength falls at a range of 700 nm where a tissue has a high transparency. Such radiation can reach deep-lying layers and produces less damage in tissues [3].

The application of OCAs may prove to be particularly relevant for enhancing two-photon microscopy [86], since it has been shown that the effect of scattering drastically reduces penetration depth to lesser depths than that of the equivalent single photon fluorescence, while largely leaving resolution unchanged [73]. This happens mostly owing to excitation beam defocusing (distortion) in the scattering media. On the other hand, this technique is useful in understanding the molecular mechanism of tissue optical clearing upon immobilization and dehydration.

The first demonstration of two-photon in-depth signal improvement using the optical immersion technique with hyperosmotic agents, such as glycerol and propylene glycol, both in anhydrous form, and aqueous glucose solution, was performed by Cicchi and colleagues in ex vivo experiments with human dermis [86]. Such improvements were obtained within a few minutes of application of three different agents, among which glycerol was the most efficient with respect to saturation level, but also the slowest. Propylene glycol was similarly efficient, whereas glucose was the worst, but diffuses three-times faster than glycerol and five-times faster than propylene glycol.

The effect on deeper layers is greater because of the cumulative effect of the reduction in scattering in the superficial layers of the tissue sample, which provides less attenuation of the incident and detected fluorescent light. The better focusing (less focused beam distortion) is achieved in less scattering media [86].

It was shown that joint confocal/two-photon microscopy provides a noninvasive way to microscopically examine the scaffold structure, which would be a valuable tool to studying biomaterials and their interactions with the molecule/cell of interest within the scaffold in an integrated fashion [73]. The integration of FocusClear-mediated optical clearing, confocal/two-photon microscopy and 3-D image rendering provided a useful approach to microscopically examine the scaffold structure. Furthermore, it was mentioned that FocusClear was significantly better than DMSO and glycerol in achieving a higher percentage of transmittance at 488, 543 and 633 nm, and fluorescent emission at 505 nm.

Additional morphological information is provided by combination of second harmonic generation (SHG) microscopy with two-photon excitation fluorescence microscopy [87]. Nonlinear laser imaging can be improved at tissue optical clearing, but it requires further investigations.

**Nonlinear microscopy**

Much like multiphoton-excited fluorescence microscopy, nonlinear microscopy provides intrinsic optical sectioning owing to the required high peak power. Furthermore, the near-infrared wavelengths that are typically used allow for penetration of several hundred microns into highly turbid tissues. SHG arises from the highly organized assembly of molecules in noncentrosymmetric environments, and has been used to image cellular membranes with great specificity and provide data regarding the organization and symmetries of structural protein arrays in tissues [88]. In its turn, the third harmonic generation is highly sensitive to changes in refractive index, and can be used to resolve interfacial regions of cells and tissues [89].

Owing to its coherent nature, the SHG wave is primarily emitted in the forward direction. For thicker, turbid, highly scattering specimens, a fraction of the SHG is backscattered. The bulk of the SHG is still forward directed. Much of the structural information encoded in the SHG signal in the forward direction will be lost in the backward signal owing to the multiple scattering events. However, the backscattered mode of SHG allows imaging of the top layers of intact skin or whole animals that cannot be performed in a transmitted geometry [90].

To achieve greater in-depth imaging of specimens of skeletal muscle tissue and mouse tendon (collagen based) by 3D SHG imaging microscopy in the forward direction mode, optical clearing with glycerol was applied [90]. It was found that treatment with 50% glycerol results in a 2.5-fold increase in the SHG imaging depth. The improvement of SHG imaging at tissue immersion was obtained owing to more effective packing of muscle bundles.

It was also shown that the axial attenuation of the forward SHG signal decreases with the increase of glycerol concentration (25, 50 and 75%) [91]. On the contrary, backward SHG signal decreases dramatically with clearing owing to negligible scattering and change of local density of SHG-producing dipoles. This response is resultant from the combination of the primary and secondary filter effects on the SHG creation and propagation, respectively [91].

Collagen fibers produce a high second harmonic signal and can be imaged inside skin dermis with SHG microscopy [92]. Yeh et al. [30] and Zhu et al. [33] demonstrated that for the case of collagen (tendon samples and engineered tissue models), the optical clearing process using high concentrated glycerol (13 M) resulted in a significant decrease in the backscattered SHG intensity for the specimens (FIGURES 3A & B). Conversely, improved transparency was observed in the transmitted light images. Transmission spectra were returned back to initial state after tissue rehydration by the application of phosphate-buffered saline [30]. Since the SHG is a more sensitive probe of the underlying structure in the array, total recovery of collagen structure was not observed. These results suggest that the collagen assembly was adversely affected.

In recent in vivo studies, glycerol was injected into rat skin for 10 min and tissue samples were immediately cut for SHG imaging; collagen fibers can be clearly seen and their dissociation or swelling was observed after injection of glycerol in concentrations from 20 to 75% for a short period of time [33] (FIGURES 3C–E). Only for 75% glycerol, diameters of fibers were smaller than these in the control group, while for other groups, the changes were...
not significant. The authors hypothesized that the change in the fiber diameter was due to tissue dehydration. The bulk summary change of collagen diameters was weaker than the change of dermal thickness [33].

It is possible that SHG and optical clearing may provide an ideal mechanism to study morphology and physiology in highly scattering tissues. However, the problem of the change of SHG signal at the optical clearing of fibrous tissue, such as skin, sclera and others, requires further research.

**Polarized microscopy**
The kinetics of the polarization properties of the tissue sample at immersion can be easily observed using an optical scheme with a white light source and a tissue sample placed between two parallel or crossed polarizers. Practically all healthy connective and vascular tissues show the strong or weak optical anisotropy typical for either uniaxial or biaxial crystals [93]. Pathological tissues, as a rule, show isotropic optical properties because of irregularities of tissue structure (more chaotic cell structures and blood vessel supply network) [94]. Owing to multiple scattering, the polarization properties are lost in turbid tissues. These very informative properties can be retained through the use of optical clearing. Reduction of the scattering at optical immersion makes it possible to detect the polarization anisotropy of tissues more easily and to separate the effects of light scattering and intrinsic birefringence on the tissue polarization properties. It is also possible to study birefringence of form with optical immersion, but when the immersion is strong, the average refractive index of the tissue structure could be close to the index of the ground media, and the birefringence of form may also be reduced and be too small to be detected [15].

At the reduction of scattering, tissue birefringence can be measured more precisely. In particular, the birefringence of form and material can be separated. For example, in a translucent human scleral sample that is impregnated with a highly concentrated glucose solution (~70%), the measured optical anisotropy \( \Delta n = (n_t - n_o) \), where \( n_t \) and \( n_o \) are the refractive indices of extraordinary and ordinary rays, respectively, was equal to approximately \( 10^{-2} \) [15].

Polarization response in SHG imaging microscopy is a valuable method to quantify aspects of tissue structure, and may be a means to differentiate normal and diseased tissues [92,93]. The SHG intensity as a function of laser polarization and the SHG signal anisotropy in marine tendon and striated muscle over a depth range of 200 µm has been measured [95]. It was shown that the laser polarization is highly randomized in the uncleared tissues at depths corresponding to only two to three scattering collisions (~50 µm). The depolarization of the laser beam also leads to randomization of the SHG signal anisotropy as it is created over a number of polarization states. In strong and normal tissues, both polarization signatures are significantly retained through approximately 200 µm of tissue thickness following treatment with 50% glycerol. Moreover, the measured polarization responses for both tendon and striated muscle are consistent with the extent of reduction of the respective scattering coefficients upon clearing.

The method can be applicable to imaging of connective disorders, as well as cancer, through several hundred microns of extracellular matrix [95].

**Ultramicroscopy**
An approach that allows for imaging of whole fixed mouse brains by ultramicroscopy is described [96]. Light diffraction on the objects with sizes less than half of the light wavelength lies at the base of ultramicroscopy. Owing to the relatively large size of the sample, the excitation light is attenuated as it penetrates the sample. To compensate for this attenuation, two light-sheets are generated that illuminate the sample from the opposing directions [97]. The authors modified ultramicroscopy by combining it with optical clearing of tissue. It was shown that this new technique allows for optical sectioning of fixed mouse brains with a
cellular resolution, and can be used to detect single green fluorescent protein-labeled neurons in excised mouse hippocampi. 3D images of dendritic trees and spines of populations of neurons in isolated hippocampi were obtained. This method is ideally suited for high-throughput phenotype screening of transgenic mice, and thus will benefit from the investigation of disease models [96]. Applications of ultramicroscopy include the imaging of cleared mouse organs and whole-mount embryos, adult Drosophila fruit flies and other fixed tissues a few millimeters in size [98].

As OCAs for ultramicroscopy and some other applications, the following preparations with different refractive indices were tested: 75% verografin aqueous solution (1.43), 1,3-propanediol (1.44), 75% iothalamate meglumine natrium (1.44), glycerol (1.47), methylsalicylate (1.53) and a mixture of benzyl alcohol/benzyl benzoate (Murray’s clear) (1.55) [99]. An increase in cell optical permeability involved freeze-thawing and modified Dent’s bleach (methanol/DMSO/30% H2O2). The most effective OCAs were considered to be Murray’s clear and 75% iothalamate meglumine natrium [99].

Selective plane illumination microscopy
Large, living biological specimens present challenges to existing optical imaging techniques because of their absorptive and scattering properties. Selective plane illumination microscopy (SPIM) was developed to generate multidimensional images of samples up to a few millimeters in size [100]. The system combines 2D illumination with orthogonal camera-based detection to achieve high-resolution, optically sectioned imaging throughout the sample, with minimal photodamage and at speeds capable of capturing transient biological phenomena.

In SPIM, the excitation light is focused by a cylindrical lens to a sheet of light that illuminates only the focal plane of the detection optics, so that no out-of-focus fluorescence is generated (optical sectioning). The net effect is similar to that achieved by confocal laser scanning microscopy. However, in SPIM, only the plane currently observed is illuminated and therefore affected by bleaching. Therefore, the total number of fluorophore excitations required to image a 3D sample is greatly reduced compared with the number in CM. SPIM can also be used to image the internal structures of relatively opaque embryos [100].

In comparison with ultramicroscopy, where the fixed sample is immersed in a clearing solution, and illuminated and imaged from outside the chamber, for SPIM the live sample is most often immersed in an aqueous medium. In order to control the position of the sample precisely with respect to the light-sheet and the detection lens, large samples such as zebrafish or Drosophila embryos are embedded in a transparent gel, such as agarose [97].

It is possible that the combination of tissue optical clearing and SPIM will allow SPIM applications in turbid samples.

Optical coherence tomography
Optical coherence tomography is an imaging technique that provides images of tissues with a resolution of approximately 2–10 µm or less at a depth equal or even more than 1 mm, depending on the optical properties of tissue [63]. It allows for determination of the refractive index and attenuation coefficient in layered structures in skin and other tissues.

Optical coherence tomography measures the optical back-scattering or reflectance R(z) from the tissue versus axial (in-depth) ranging distance (z). The reflectance depends on the optical properties of the tissue – that is, the absorption (µa) and scattering (µs) coefficients, or total attenuation coefficient:

\[ \text{µt} = \text{µa} + \text{µs} \]

Multiple scattering is a detrimental factor that limits the OCT imaging performances: imaging resolution, depth and localization [6].

The immersion technique is of great potential for noninvasive medical diagnostics using OCT owing to the rather small thickness of tissue layers usually examined by the OCT, which allows for fast impregnation of a target tissue at a topical application

Figure 5. Visual changes of blood vessels for in vivo rat skin before and after treatment with mixture solution of polyethylene glycol (PEG-400) and thiazone on skin surface. (A) Initial state. (B) 4 min. (C) 12 min. Reproduced with permission from [106].
of an immersion agent [6,7]. It has been demonstrated that the body’s interior tissues, such as the blood vessel wall, esophagus, stomach, cervix and colon, can be imaged to a depth of approximately 1–2 mm [6,7]. More effective diagnosis using the OCT with a higher penetration depth is provided by applying immersion substances [6]. Both the index matching and the localized dehydration effects can lead to the enhanced depth capability and the improvement of imaging contrast.

The possibility of in vivo diagnostics of malignant melanoma, the observation of subepidermal blisters and the control of the scattering properties of skin through the saturation of skin with glycerol and propylene glycol at their topical application were demonstrated on the basis of OCT imaging of human skin both in vitro and in vivo [7].

In OCT experiments with human skin in vitro, at topical application during 90–120 min of the combined lipophilic polypropylene glycol-based polymers and hydrophilic polyethylene glycol-based polymers, both with an index of refraction of 1.47 that closely match that of skin-scattering components in stratum corneum, epidermis and dermis, it was shown that the polymer mixture can penetrate intact skin and improve the OCT images for clearer visualization of dermal vasculature and hair follicles [101].

As an example, Figure 4 shows OCT images of the same area of a mouse embryo imaged before and 10 min after the addition of 50% glycerol (Figures 4A & B, respectively) [102]. The selected imaging areas contained heart chambers as well as more optically uniform tissues used to assess clearing effect of the introduced clearing agent. As one can see from the images, during the course of glycerol interaction with the tissue, the atrial wall became more visually defined, and the structure of the trabeculae within the ventricle became more distinguishable. Scans through the more uniform abdominal region (marked with vertical lines in Figure 4) were used to assess the degree of optical clearing. The percent of signal enhancement at the depths of 200–500 µm from the surface was calculated to be 51.5 ± 12.5% at approximately 10–15 min after addition of glycerol [102].

Optical projection tomography

Optical projection tomography (OPT) is a new approach for 3D imaging of small biological specimens. It fills an imaging gap between MRI and CM, being most suited to specimens that are from 1 to 10 mm across [103].

The ability to analyze the organization of biological tissue in 3D has proven to be invaluable in understanding embryo development, a complex process in which undergo an intricate sequence of movements relative to each other. A related goal is the mapping of gene-expression patterns onto these 3D-tissue descriptions. This information provides clues regarding the biological functions of genes, and also indicates which genes may interact with each other [104]. Since OPT can record both absorption and emission profiles, it is able to image the wealth of different staining techniques that exist to record the spatial distribution of gene activity. The most common assay for gene activity at the RNA level is still a protocol that produces a purple precipitate within the tissue. As this precipitate is not fluorescent, it cannot be imaged by CM. However, in brightfield mode, OPT can recreate the 3D distribution of this precipitate for a complete 11.5 days-post-coitum mouse embryo [103,104]. Other important applications for OPT are to help analyze normal and abnormal morphology, and to localize where labeled cells are within a tissue [103].

The most common approach for OPT imaging is to suspend the specimen in an index-matching liquid to reduce the scattering of light on the surface and reduce heterogeneities of refractive index throughout the specimen. This means that light passes through the specimen in approximately straight lines (ballistic photons), and a standard backprojection algorithm can generate relatively high-resolution images [103].

It has been shown that OPT allows for successful 3D tomographic imaging of specifically labeled structures within cleared adult mouse organs without autofluorescence-induced artifacts [105].

Blood microvessel imaging

The skin optical clearing makes it possible to observe dermal blood vessel through the intact skin (e.g., Figure 5) [9,106]. Figure 5 shows the result of the action of a mixture of polyethylene glycol (PEG-400) with the penetration enhancer thiazone, topically applied to rats’ skin in vivo. It is shown that the skin becomes transparent after 12 min and the vessels are clear [106]. The method is convenient, cheap and simple for in vivo monitoring of microcirculation. It may be useful for the study of the structure and function of blood microvessels, diameters of arterioles and venules, capillary density, bifurcation angles, and so on.

Glucose sensing

NIR technique

Measurements of blood glucose and its regulation are necessary for patients with disorders of their carbohydrate metabolism, particularly caused by diabetes mellitus. Over the past decades, noninvasive blood glucose monitoring has become an increasingly important topic in the realm of biomedical engineering. In particular, the introduction of optical approaches has brought exciting advances to this field [77,107]. NIR spectroscopy has been known to have potential in realizing noninvasive blood glucose monitoring. The NIR region is ideal for the noninvasive measurement of human body compositions because biological tissue is relatively transparent to the light in this region, so-called therapeutic window. The molecular formula of glucose is C₆H₁₂O₆, and several hydroxyl and methyl groups are contained in this structure. They are main hydrogen functional groups, whose absorption occurs in the NIR region. The second overtone absorption of the glucose molecule is in the spectral region between 1100 and 1300 nm, and the first overtone absorption of the glucose molecule is in the region of 1500–1800 nm [77]. In the range 1400–1500 nm, there is a peak, corresponding to the absorption peak of water [108]. This information provides the theoretical basis for the measurement of blood glucose using NIR spectroscopy [77]. Moreover, tissue optical clearing can be helpful for more contrast visualization of glucose bands in tissue reflectance spectra. For example, selection of an OCA without the peak
in the range of approximately 1600 nm can provide dehydration of tissue and, thereby, decreasing of the water peak, as well as the light-scattering suppression. Thus, the glucose peak at 1600 nm can be better differentiated.

One critical difficulty associated with in vivo blood glucose assays is an extremely low signal-to-noise ratio of glucose peak in an NIR spectrum of human skin tissue. Therefore, the main problem of the NIR glucose monitoring is to build the calibration models [66,77].

The concept of noninvasive blood glucose sensing using the scattering properties of blood is an alternative to the spectral absorption method. The method of NIR frequency-domain reflectance techniques is based on a change in glucose concentration, which affects the refractive index mismatch between the interstitial fluid and tissue fibers, and hence \(\mu_s\). The refractive index \(n\) of the interstitial fluid modified by glucose is defined by the equation [78]: \[ n_{glw} = n_w + 1.515 \times 10^{-6} \times C_{gl} \] where \(C_{gl}\) is glucose concentration in mg/dl and \(n_w\) is the refractive index of water [109]. As the subject’s blood glucose rose, the \(\mu_s\) decreased. Key factors for the success of this approach are the precision of the measurements of the reduced scattering coefficient and the separation of the scattering changes from absorption changes, as obtained with the NIR frequency-domain spectrometer [78]. Evidently, other physiological effects related to glucose concentration could account for the observed variations of \(\mu_s\) and, as was mentioned earlier, the effect of glucose on the blood flow in the tissue may be one of the sources of the errors at \(\mu_s\) measurements.

**OCT technique**
The OCT method can also be used for glucose monitoring in tissues. Since the OCT technique measures the in-depth distribution of reflected light with a high spatial resolution, changes in the in-depth distribution of the tissue scattering coefficient and/or refractive index mismatch are affecting the OCT signal parameters – slope and amplitude. Both free blood glucose perfusion via blood capillaries and follow-up diffusion of glucose in tissues induce local changes of optical properties (scattering coefficient and refractive index); thus, one can monitor and quantify the diffusion process by depth-resolved analyses of the OCT signal recorded from a tissue site [64,65,79–81]. Physiological glucose levels are in the range of 3–30 mM, which are far below the level of making the tissue transparent. However, OCT is capable of detecting photons backscattered from different layers in the sample. Thus, changes in the scattering properties can be studied at different depths in the sample [81]. Therefore, a high sensitivity of the OCT signal from living tissues to glucose content allows one to monitor its concentration in the skin at a physiological level [79].

**Photoacoustic technique**
Both in vitro and in vivo studies have been carried out in the spectral range of the transparent ‘tissue window’, of approximately 1–2 \(\mu m\), to assess the feasibility of photoacoustic spectroscopy (PAS) for noninvasive glucose detection [82]. The photoacoustic signal is obtained by probing the sample with monochromatic radiation that is modulated or pulsed. Absorption of probe radiation by the sample results in localized short-duration heating. Thermal expansion then gives rise to a pressure wave, which can be detected with a suitable transducer. An absorption spectrum for the sample can be obtained by recording the amplitude of generated pressure waves as a function of probe beam wavelength [82]. The pulsed photoacoustic signal is related to the properties of turbid medium by the equation [76,82]:

\[
PA = k(E_0 \mu_s/C_p) E_0 \mu_{\text{eff}}
\]

where \(PA\) is the signal amplitude, \(k\) is the proportionality constant, \(E_0\) is the incident pulse energy, \(\beta\) is the coefficient of volumetric thermal expansion, \(\nu\) is the speed of sound in the medium, \(C_p\) is the specific heat capacity and \(n\) is a constant between one and two depending on the particular experimental conditions.

\[
\mu_{\text{eff}} = \sqrt{b(C_p + \mu_s/C_p)}
\]

In the PAS technique, the effect of glucose can be analyzed by detecting changes in the peak-to-peak value of laser-induced pressure waves [110].

The investigations demonstrated the applicability of PAS to measurement of glucose concentration [110]. The great percentage of change in the photoacoustic response was observed in the region of the C-H second overtone at 1126 nm, with a further peak in the region of the second O-H overtone at 939 nm [82]. In addition, the generated pulsed PA time profile can be analyzed to detect the effect of glucose on tissue scattering, which is reduced by increasing glucose concentration [79,83].

**Raman spectroscopy**
Raman spectroscopy (RS) can provide potentially rapid, precise and accurate analysis of OCA concentration and biochemical composition. RS provides information regarding the inelastic scattering, which occurs when vibrational or rotational energy of target molecules is exchanged with incident probe radiation. Raman spectra can be utilized to identify molecules such as glucose, because these spectra are characteristic of variations in the molecular polarizability and dipole momentum. Enejder et al. accurately measured glucose concentrations in 17 nondiabetic volunteers following an oral glucose tolerance protocol [83].

In contrast to infrared and NIR spectroscopies, RS has a spectral signature that is less affected by water, which is very important for tissue study. In addition, Raman spectral bands are considerably narrower (typically 10–20 \(\text{cm}^{-1}\) in width [111]) than those produced in NIR spectral experiments. The RS also has the ability for simultaneous estimation of multiple analytes, requires minimum sample preparation and would allow for direct sample analysis [112]. Like infrared absorption spectra, Raman spectra exhibit highly specific bands that are dependent on concentration. As a rule, for Raman analysis of tissue, a spectral region between 400 and 2000 \(\text{cm}^{-1}\), commonly referred to as the ‘fingerprint region’, is employed. Different molecular vibrations lead to Raman scattering in this part of the spectrum. In many cases, bands can be assigned to specific
molecular vibrations or molecular species, aiding the interpretation of the spectra in terms of biochemical composition of the tissue [112].

Owing to the reduction of elastic light scattering at tissue optical clearing, more effective interaction of a probing laser beam with the target molecules is expected. OCAs increase the signal-to-noise ratio, reduce the systematic error incurred as a result of incompletely resolved surface and subsurface spectra, and significantly improve the Raman signal [112].

Raman spectroscopic diffuse tomographic imaging (785-nm excitation wavelength) has been applied in order to image the composition of bone tissue ex vivo within an intact section of a canine limb. Glycerol was applied for optical clearing [113]. The effect of optical clearing with glycerol on the Raman spectra of bone tissue acquired transcutaneously on tibiae from mice was also studied [114]. Glycerol reduced the noise in the raw spectra and significantly improved the cross-correlation between the recovered bone factor and the exposed bone measurement in a low signal-to-noise region of the bone spectra.

**Monitoring of drug delivery**

Studying the diffusion of drugs within tissues is vital for the estimation of a drug-dose delivery into a targeted area, the time it takes for the drug to have an effect, and so on. Since the refractive index of aqueous solutions of some drugs is higher than that of the interstitial fluid, the penetration of the drugs into tissues has to induce optical clearing of the tissues. The results of the experiments have shown that penetration of some drugs into scleral tissue leads to the decrease of scleral reflectance owing to optical immersion [115,116]. Thus, the diffusion coefficient of the drugs in fibrous tissue can be estimated by the method based on the measurements of temporal changes of optical properties of a tissue (transmittance or reflectance) caused by temporal refractive index matching [23].

The permeability coefficient for drug solution in tissue layers can be also measured using OCT methods. An increase in refractive index of the interstitial fluid and corresponding decrease in scattering can be detected as a change in a slope of the curve of the OCT signal. In the course of drug diffusion, an increase in local in-depth drug concentration results in a decrease of OCT signal slope [7,64].

**Therapeutic applications**

*In vivo* control of tissue optical properties can be very important for some therapeutic applications. In particular, transscleral laser operation, in which the retina and ciliary body are irradiated by a laser beam directly through sclera, is promising in ophthalmology for the treatment of glaucoma, myopia and other diseases [117].

Both scattering and absorption of different layers of the eye under action of 40% glucose solution have been simulated by the Monte Carlo method [9]. This model has shown that the mean increase of the fraction of photons absorbed in retina during the clearing of sclera is approximately 30%. Thus, scleral optical clearing may provide more precise and effective coagulation of retinal pigmented epithelium and choroid layers.

Experiments have shown that OCAs (in particular, glycerol) can be used for significantly reducing the radiant exposures required for cutaneous and epidermal blood vessel coagulation [9,10,106,118], and tattoo removal [119,120].

In many cases, targeted deep blood vessels in a lesion are not sufficiently heated by incident light owing to competition from absorption and scattering by other tissue constituents. The skin optical clearing method was proposed to make it possible to observe dermal blood vessels through the intact skin [9,106]. Subcutaneous radiant exposures for vessels treated with glycerol were typically several folds lower than untreated vessels. For example, arterioles in the 80–110-µm diameter range in untreated skin had radiant exposure values approximately 12 J/cm², compared with approximately 2 J/cm² in glycerol-treated cases [9].

For the estimation of effectiveness of laser radiation delivery to the area of the target absorber localization, the computer simulation of the alteration of skin optical properties was carried out by the Monte Carlo modeling [120]. It was shown that the use of OCA allows same results as without optical clearing at the lesser laser energy to be achieved. For example, at tattoo removal, the density of laser energy can be reduced to 50–60% depending on the target area localization depth in...
the blue-green spectral range, to 30–40% in the red spectral range, and to 10–20% in the NIR spectral range. Thus, optical clearing can allow one to avoid thermal damage of surrounding skin with the similar damage of the absorbing target [120]. It was shown that the use of glycerol in conjunction with microneedling and tissue compression can deliver more photons to deeply located chromophores by effectively improving the laser-beam profile in tissue [118].

It should be noted that reduction of the scattering should be provided for a short period of time, otherwise very low scattering in the area of the absorber will lead to the reduction in the amount of absorbed energy by a target. Fortunately, for many tissues it is very difficult to make bulk tissue totally transparent, but the clearing of the upper layers of tissues is often sufficient to irradiate the absorbing target in an optimal way.

**Toxicity of optical clearing**

Widely used hyperosmotic fluids (e.g., glucose, glycerol and propylene glycol) are generally nontoxic agents. However, they can induce local hemostasis, and even tissue necrosis, with prolonged application of highly concentrated OCAs, due to the induction of osmotic stress.

It was shown that the topical application of 75% glycerol solution on the mesenteric microvessels of a rat *in vivo* during the initial period of 1–3 s led to slowing down of blood flow in all microvessels (arteries, venules and capillaries) [42]. After 20–25 s, the stasis appeared and vessels were dilated on average by 30%. Simultaneously, the red homogeneous contents in the lumen of the majority of vessels were visualized. This fact is most probably a evidence of intravascular hemolysis. By 6 min, the walls of the vessels cannot be seen. Such changes of microcirculation were exactly local within the area of glycerol application. At a short distance (400–500 µm) from this area, only a slight decrease in blood flow was registered.

With glucose concentration decreasing, the toxic effect also decreased, and hemostasis was not observed [42].

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**Table 1. Increasing light penetration depth and imaging contrast induced by optical clearing agents in different diagnostic techniques.**

<table>
<thead>
<tr>
<th>Diagnostic technique</th>
<th>Tissue</th>
<th>OCA, refractive index</th>
<th>Spectral range (nm)</th>
<th>Penetration depth increasing</th>
<th>Relative contrast</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confocal microscopy</td>
<td>Dehydrated murine fat, muscle, heart and brain</td>
<td>Murray’s clear, 1.55</td>
<td>334–799.3</td>
<td>~35-fold</td>
<td>–</td>
<td>[72]</td>
</tr>
<tr>
<td>3D SHG imaging microscopy (forward mode)</td>
<td>Murine muscle</td>
<td>50% glycerol, 1.395</td>
<td>Excitation – 890, detection – 445</td>
<td>2.5-fold</td>
<td>–</td>
<td>[90]</td>
</tr>
<tr>
<td>Polarization SHG imaging microscopy (forward mode)</td>
<td>Murine tendon and muscle</td>
<td>50% glycerol, 1.395</td>
<td>Excitation – 890, detection – 445</td>
<td>4-fold</td>
<td>–</td>
<td>[95]</td>
</tr>
<tr>
<td>OCT</td>
<td>Mouse embryo</td>
<td>Polypropylene glycol- and polyethylene glycol-based prepolymer mixture, 1.47</td>
<td>1310</td>
<td>4.5-fold</td>
<td>–</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td><em>In vivo</em> human skin</td>
<td>Polypropylene glycol- and polyethylene glycol-based prepolymer mixture, 1.47</td>
<td>1305</td>
<td>1.2-fold</td>
<td>–</td>
<td>[101]</td>
</tr>
<tr>
<td></td>
<td>Whole blood twice diluted with saline</td>
<td>6.5% glycerol, 1.34</td>
<td>820</td>
<td>2.4-fold</td>
<td>–</td>
<td>[38]</td>
</tr>
<tr>
<td>Spectrophotometry</td>
<td>Bovine serum albumin gel</td>
<td>FocusClear, 1.46</td>
<td>488–633</td>
<td>46.3-fold</td>
<td>–</td>
<td>[73]</td>
</tr>
<tr>
<td></td>
<td>Human bone</td>
<td>Anhydrous glycerol, 1.47</td>
<td>1400–2000</td>
<td>1.2-fold</td>
<td>–</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td>Human dura mater</td>
<td>0.16 g/ml aqueous mannitol solution, 1.357</td>
<td>400–700</td>
<td>1.3-fold</td>
<td>–</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td><em>In vivo</em> rabbit sclera</td>
<td>40% glucose, 1.39</td>
<td>568, 610</td>
<td>–</td>
<td>&gt;2-fold</td>
<td>[8]</td>
</tr>
</tbody>
</table>

OCA: Optical clearing agent; OCT: Optical coherence tomography; SHG: Second harmonic generation.
Long-term effects on blood vessels were investigated by observing the function and the development of blood vessels in chick chori- oallantoic membrane. The results have shown that at glycerol application, the blood flow velocity was recovered to different extents, and new blood vessels developed at 2 days. Glucose induced slow changes in blood flow or vessels. However, most blood vessels were blocked and no new blood vessels developed at 2 days [43].

The study of kinetics of optical clearing of rat skin in vivo at 84.4% glycerol intradermal injection have shown transition from the oxygenated form of Hb to the deoxygenated form 15 min after injection [121]. It was suggested that this effect was related to local hemostasis of skin vessels caused by the high concentration of glycerol.

The in vivo effect of anhydrous glycerol on cutaneous vasculature has also been studied by Doppler OCT [122]. An important finding of that study was that the effect of glycerol on vessels was reversible with rehydration; when the skin was rehydrated using saline applied to the skin site, flow in arterioles and venules returned to physiologic values and remained at those values.

As mentioned earlier, the irreversible changes of collagen structure under the action of pure glycerol are possible [30]. However, this effect is observed in tendon and model samples that have well-ordered fibrillar organization. After glycerol removing and rehydration of the samples, the structure does not reshape totally. In softer skin, this effect can be reversible [30]. In vivo experiments have shown that a glycerol solution of 75% does not induce any loss of collagen organization [33].

In a number of studies some side effects of optical clearing in the format irritation and edema, were noted [8,24,27,28,33]. However, further investigations are still needed in order to find the optimal and safest concentration of agent that can be applied to living tissues.

Conclusion
This article demonstrates some specific features of tissue optics and light-tissue interaction. It shows that administration of OCAs allows scientists to effectively control the optical properties of tissues. The control leads to the essential reduction of scattering, therefore allowing increasing efficacy for the application of different optical imaging and spectroscopic (optical biopsy) techniques for medical purposes. The immersion technique also has great potential for a number of therapeutic and surgical methods using laser-beam action on a target area hindered within the tissue.

Expert commentary
The future of medicine is focused on the development and introduction of advanced optical technologies, in particular, with receiving more valuable information from spectroscopic and polarization measurements, CM, ultramicroscopy and SPIM, optical projection and optical coherence tomography, as well as from nonlinear spectroscopies such as two-photon fluorescence and SHG techniques. The critical points for the wide biomedical application of these progressive novel techniques are limitations in penetration depth, image contrast and spatial resolution. In this context, the authors have shown that the increase in the optical transparency of tissues and blood owing to immersion optical clearing can substantially improve the penetration depth, image contrast and spatial resolution of optical diagnostic methods. Table 1 shows some examples of increasing both light penetration depth and imaging contrast at optical clearing.

Recent technological advancements in the photonics industry have led to the rise of interest in optical imaging and spectroscopic technologies, and real progress toward the development of noninvasive sensing of drugs and metabolic analytes in tissues and blood. Optical noninvasive methods of blood glucose monitoring based on light-scattering phenomena conditioned by refractive index matching/mismatching, and dynamic RBCs aggregation/disaggregation, have potential advantages over the methods based on absorption measurements, for which the main problem lies in building an adequate calibration model. However, these advances should be realized in the form of practical techniques, which require further investigations.

Evaluation of diffusivity and permeability coefficients of immersion agents and drugs delivered through tissues is also based on the measurements of temporal changes of optical properties of a tissue. These data are of great importance for the accurate estimation of drug delivery concentration and rate into the target tissue region, time of drug action, and so on.

Sometimes, Hb can be used as a specific blood optical clearing agent. Local hemolysis with production of nontoxic amounts of free Hb in the vicinity of the optical head of endoscopic OCT, or another endoscopy-spectroscopic system, was demonstrated as a potential robust tool for improvement of images of vessel wall lesions owing to increase of light penetration through the bloodstream.

The reduction of tissue light scattering not only gives a better image quality and precision of spectroscopic information, but also substantial improvement of the quality of the irradiating light beam and its sharp focusing within tissue. The corresponding increase of efficiency of laser radiation delivery to the target region allows one to provide more precise damage of tissue lesions, and to avoid thermal damage of surrounding healthy tissues.

Five-year view
Following the development of the immersion refractometry method applicable to cells, in 1955 Barer et al. pioneered the optical clearing of a cell suspension by a protein solution having the same refractive index as the cytoplasm of the cells [123]. In the early 1990s, the immersion optical clearing method was applied to tissues, since that time we have seen growing interest in this method, which could be associated with the developments of the optical and laser technologies for biology and medicine (Figure 6). In the last 10 years, numerous investigations related to the decreasing of tissue scattering properties owing to administration of immersion agents were carried out [3,6,7,124–126]. The rapid upsurge of publications in this period was caused by the development of optical technologies applicable for noninvasive diagnostics, therapy and surgery of different diseases. Optical clearing is the easiest and most low-cost way for substantially increasing the effectiveness of most of the optical technologies.
In the near future, we believe that increasing numbers of new research groups will appear, with their main focus being the search for and design of effective and safe OCAs, their diffusion providers, and enhancers for delivery through stratum corneum and mucous. More efficient and biocompatible tools based on physical principles, such as sonophoresis, electro- and light-delivery, fractional ablation, needle-free jet injection and other delivery techniques, will be designed and applied in clinics for OCA delivery.

Noninvasive glucose sensing could be an attractive area of application of basic research on the interaction of physiological and elevated diabetic concentrations of glucose with tissues and blood, diffusivity of glucose molecules and impact on hemoglobin, collagen, albumin and other proteins.

We are expecting the appearance of new multimodal clinical optical imaging systems and technologies with the in-built optical clearing procedure, as has already happened for the opposite techniques, such as tissue ‘whitening’, where acetic acid is used to make tissue more turbid to increase the output of fluorescence techniques, such as tissue ‘whitening’, where acetic acid is used to make tissue more turbid to increase the output of fluorescence of the superficial tissues layers in cervical studies.

At the moment, only a few tissue are studied more or less fully, and these are skin, eye sclera and cerebral membrane (dura mater); obviously, more intensive studies are needed for these tissues and for a number of other soft and hard tissues that have been studied nonsystematically or not yet studied at all. Among soft tissues, the mucosal tissue of different organs, brain, muscle, myocardium, vessel wall, lymph node, stomach, esophagus, kidney and liver tissues are of great interest to be cleared with light diagnostic therapy or surgery. For hard tissues, optical clearing of cranial bone, tooth enamel, dentin and cement, cartilage, tendon and ligament could be important to provide more efficient laser therapy and surgery.

Therefore, the tissue optical clearing method has great future prospects owing to its simplicity, low cost and low risk.

**Key issues**

- The optical immersion technique is based on the impregnation of a tissue by a biocompatible chemical agent that leads to decreasing of light scattering in tissue.
- The main mechanisms of light scattering reduction induced by optical clearing agents are: dehydration of tissue constituents; partial replacement of the interstitial fluid by the immersion substance; and structural modification or dissociation of structured proteins, such as collagen. At the clearing of tissues in vivo, when clearing agents are not highly concentrated and have limited action time, the first two mechanisms are more probable.
- Reduction of tissue scattering owing to administration of immersion agents leads to improved signal detection in optical coherence tomography, projection tomography, confocal and nonlinear microscopy, Raman spectroscopy, reflectance spectroscopy and other optical diagnostic techniques.
- The concept of noninvasive blood glucose sensing using the scattering properties of blood is an alternative to the spectral absorption method.
- Drug delivery through epithelial and fibrous tissues can be monitored by the measurements of temporal changes of optical properties of a tissue (transmittance or reflectance) caused by temporal scatterer refractive index matching/mismatching. More precise spatially-resolved measurements could be provided by optical imaging technique and confocal microscopy.
- Theoretical analysis and experiments have demonstrated that optical clearing can be used for significant reduction of the radiant exposures in transscleral and transcutaneous laser surgery.
- The immersion optical clearing method has good future prospects for the near future.

**References**

Papers of special note have been highlighted as:

- of interest
- **of considerable interest**


- Discusses the results of theoretical and experimental investigations into photon transport in tissues. Describes methods for solving direct and inverse scattering problems for random media with multiple scattering, and quasi-ordered media with single scattering, in order to model different types of tissue behavior. This book provides the optical parameters of various tissues.

No writing assistance was utilized in the production of this manuscript.

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This book provides the basic principles, results, advantages, limitations and future of the optical immersion method applied to clearing of the naturally turbid biological tissues and blood.


The first paper that presents optical clearing of eye sclera in vivo.


Agrba PD, Kirilllin MY, Abelechiv AI, Zagaynova EV, Kamensky VA. Compression as a method for increasing the informativity of optical coherence tomography of biotissues. Optics and Spectroscopy 107(6), 853–858 (2009).


Initial work in tissue optical clearing.


Initial work in tissue optical clearing.


First paper that shows simulation of blood clearing by local hemolysis.


Presents analysis of short-term and long-term effects of different optical clearing agents on blood vessels.


Shows the influence of various alcohols on skin optical clearing. This is important because alcohols are enhancers of diffusion.


Gerger A, Koller S, Kern T et al. Diagnostic applicability of in vivo confocal laser scanning microscopy in melanocytic...


First paper that suggested using skin optical clearing for increasing the probing depth in the method of confocal microscopy.


These authors were the first who suggested using skin optical clearing for contrast and depth enhancement in two-photon microscopy.


These authors suggested using optical clearing for improvement of contrast in second harmonic generation imaging of muscle.


Sharpe J, Ahlgren U, Perry P et al. Optical projection tomography as a tool for 3D


• Gives a complete, state-of-the-art treatise on the design and applications of noninvasive optical methods and instruments for glucose sensing.


• Suggests the method of drug delivery monitoring and evaluation of drug diffusion coefficient from reflectance.


• Analyzes mechanisms of skin optical clearing by glyceral ex vivo and in vivo.


• Important for understanding the fundamentals of optical clearing.


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