

Nonlinear Optics of Skin: Enhancement of Autofluorescence and Second Harmonic Generation Signals by Immersion Optical Clearing

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Abstract. Multiphoton microscopy and especially two-photon microscopy are actively used in dermatology for the diagnosis and analysis of skin diseases. The typical skin probing depth for these methods is limited to 150–200 μm due to the strong scattering properties of skin. The application of the optical clearing method to biological tissues makes it possible to control their optical properties, namely light scattering, by matching the refractive indices of the structural components of tissues. Reducing the scattering of the upper layers of tissue increases the depth of probing for any method of optical imaging by increasing the intensity of the optical signal recorded from the depth up to 6.6 times. This paper presents a brief review of the methods of nonlinear optics used to assess the condition of the skin, discusses the possibility of improving the efficiency of diagnosing skin diseases through the use of the optical clearing method. In particular, this paper discusses the enhancement of the intensity of autofluorescence and second harmonic generation signals during two-photon microscopy of the skin. © 2023 Journal of Biomedical Photonics & Engineering.

Keywords: skin; optical clearing; fluorescence; second harmonic generation; imaging; stratum corneum; dermis; collagen.

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1 Introduction

Fluorescence is one of the basic mechanisms of interaction between optical radiation and biological objects. In general, fluorescence corresponds to a spin-allowed optical transition between singlet or triplet levels characterized by a short lifetime (10^{-11} – 10^{-6} s) and relatively large quantum yield [1]. The fluorescence methods of optical imaging are currently become one of

the most popular and promising research directions for non-invasive diagnosis and monitoring of biological tissues, since their application makes it possible to obtain functional images of the studied objects and analyse the obtained data at the molecular level with high resolution [2, 3].

One of the most effective methods of fluorescence optical imaging is multiphoton microscopy (MPM) and,

particularly, two-photon microscopy (TPM) method, basing on a nonlinear two-photon fluorescence excitation process [4]. TPM uses both scattered and ballistic fluorescence photons excited at a wavelength longer than the second harmonic generation (SHG) wavelength considering the used laser excitation source and registered with a wide-aperture photodetector. TPM approach allows for three-dimensional analysis of chromophores distribution due to their excitation by a laser source in the near-infrared (NIR) region. Since biological objects have high transparency in the infrared spectral region, the excitation radiation reaches the deepest layers. Also, the low absorption in the infrared range reduces damage to biological objects. In case of two-photon excitation at 760 nm, fluorescence emission lies in the visible spectral range (410–680 nm) [5]. Recorded signal comes from focal volume of microscope and practically does not contain extraneous background noise.

TPM method combined with fluorescence lifetime imaging is widely used in dermatology for diagnosis and analysis of skin at the cellular level [5–12]. Nevertheless, strong light scattering properties of stratum corneum, living epidermis and dermis significantly limits penetration ability of probing light and reduce the detected signal. Therefore, analysis of fluorescence signal at depths exceeding 150–200 μm is difficult due to low fluorescence intensity as well as low signal-to-noise ratio value.

In order to increase the probing depth of optical methods in biological tissues and improve the quality of obtained images, the optical clearing (OC) method was developed [13, 14]. OC allows to control optical properties of investigated biological objects, significantly increase the efficiency of used optical imaging method, especially in case of highly scattering skin.

The current paper presents a brief review of results on investigation of OC application with the main aim to improve the skin assessment using MPM and TPM. In particular, the effects of optical clearing agents (OCAs) with different osmolarity on the intensity of SHG signal from skin is discussed.

2 Principles of Two-Photon Excitation

The theory of two-photon excitation was firstly described in 1931 by Maria Geppert-Mayer [15]. However, experimentally this effect has been confirmed only after the invention of lasers in 1961 [16]. In general, the standard one-photon fluorescence excitation is described as follows. A fluorophore in the ground state (S_0) can absorb a single photon, leading to the fluorophore excitation and transition to a higher energy state (S_1). After presence at the excited state fluorophore returns to the ground state at the same time emitting the fluorescence photon. Thus, the following condition must be satisfied for the single-photon excitation:

$$E_{S_1} - E_{S_0} = \frac{hc}{\lambda_{1p}}. \quad (1)$$

Here E_{S_1} is the energy of the fluorophore at the excited state, E_{S_0} is the energy of the fluorophore at the ground state, h is Planck's constant, c is the speed of light in a vacuum, λ_{1p} is the photon wavelength during one-photon excitation.

In the case of two-photon excitation two photons are simultaneously absorbed, and each of the photons has twice the energy of the photon comparing to one-photon excitation. Thus, since the energy of the photon is inversely proportional to its wavelength, the following condition must be satisfied for two-photon excitation:

$$E_{S_1} - E_{S_0} = 2 \frac{hc}{\lambda_{2p}}. \quad (2)$$

Here, λ_{2p} is the wavelength of the two-photon excited fluorescence.

In case of SHG excitation, simultaneous absorption of two photons leads to transition to the virtual state and subsequent emission of a photon. Thus, for two-photon excitation of SHG, the following condition must be fulfilled:

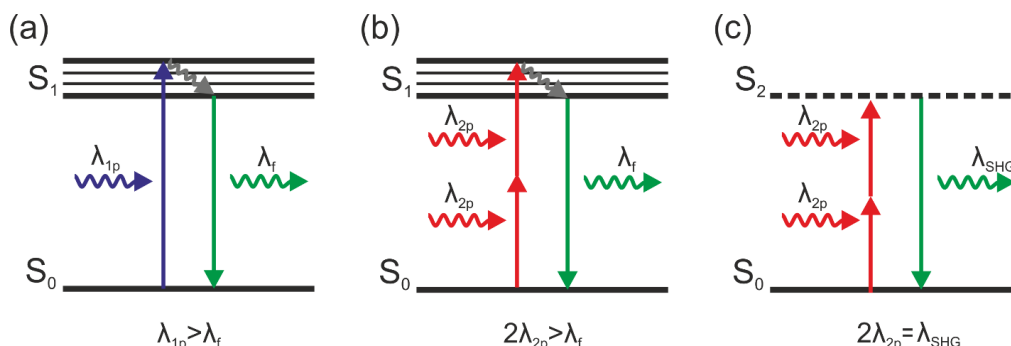


Fig. 1 Diagram showing the principle of single-photon excitation (a), two-photon excited fluorescence (b), and SHG excitation (c). S_0 is the ground state of the fluorophore; S_1 is the excited state of the fluorophore; S_2 is the virtual state of the fluorophore; λ_{1p} and λ_{2p} are the photon excitation wavelength for one-photon and two-photon excitation; λ_f is the photon excitation wavelength for one-photon and two-photon excited fluorescence; λ_{SHG} is the photon excitation wavelength for two-photon excited SHG.

$$E_{S2} - E_{S0} = 2 \frac{hc}{\lambda_{2p}}, \quad (3)$$

where E_{S2} is the energy of the fluorophore at the virtual state and λ_{2p} is the wavelength of the excitation photon during two-photon excitation of SHG. Also, two-photon excited fluorescence is generated in the entire bandwidth, while SHG has a resonant character.

Fig. 1 shows diagrams of one- and two-photon fluorescence excitation and SHG excitation processes.

As mentioned above, successful two-photon fluorescence excitation requires simultaneous absorption of two photons (the inter-event interval must be less than 10^{-18} s) [17]. Potentially, high-intensity continuous lasers can be used to increase the probability of this event. Nevertheless, in practice, pulsed sources with femtosecond pulse duration are used to work with biological objects, since they allow to reduce the tissue thermal damage due to short exposure at the same time maintaining a large number of photons in the beam [17].

The closest analogue to TPM method is confocal microscopy (CM) basing on single-photon excitation of fluorescence. However, in case of CM, fluorescence is generated not only in the focal area of the optical system, but also out of focus, which increases the noise in registered signal. Also, CM suffers from significant fluorescence photobleaching effects [18, 19] and possible phototoxicity [20]. At the same time, the use of a high-aperture lens in TPM setups allows to achieve small (sub-femtoliter) excitation volume [4]. Also, TPM allows to achieve higher probing depth and provides safer measurements due to significantly less phototoxicity and fluorescence photobleaching effects compared to CM [21].

Commercially available TPM systems allow to register autofluorescence (AF) and SHG signals by using different emission filters located before the photomultiplier tube [21, 22]. In case of skin imaging, AF signal is formed mainly by melanin, elastin, NAD(F)H, FAD and keratin. These fluorophores are unevenly distributed in skin layers. At the same time, SHG signal is formed by collagen type I molecules [22] located only in papillary and reticular layers of dermis at 50–300 μm depths. Thus, excitation and emission of light for collagen is attenuated due to high light scattering and absorption of skin epidermis. Collagen type I is not the sole source of SHG signal in the skin. Recently, the presence of crystallised urea dendriform structures in the stratum corneum of glabrous human skin has been discovered, which generate a strong SHG signal [23]. Also, AF intensity in the 400–480 nm region is attenuated due to absorption by other skin chromophores, such as, for example, hemoglobin, melanin, carotenoids [24, 25]. It should be noted that melanin presenting in the epidermis [26], is also a source of strong fluorescence signal in case of two-photon excitation [27, 28]. The JenLab TPM optical systems [21, 22], which have high spatial resolution of $< 0.36 \mu\text{m}$ (horizontally) and $< 1.7 \mu\text{m}$ (vertically), have proved to be a good tool for *in vivo* skin studies [9] allowing to obtain high quality skin images up to 150–200 μm in depth.

3 Second Harmonic Generation Signal Analysis for Skin Collagen Assessment

Relative changes in collagen type I content in dermis can be indicator of skin damage and skin aging processes [29–31]. Several studies showed that healthy skin of young people can be characterized by the interwoven structure of collagen fibers with embedded “hollows”, as well as higher concentration of collagen type I compared to skin of older people [22, 32, 33]. Various studies showed that collagen content, structural organization, and morphology can change significantly in case of various diseases (e.g. diabetes [29], Ehlers-Danlos syndrome [34, 35], Goodpasture syndrome [36], cancer [37], etc.). The use of TPM, and particularly SHG signal analysis, is an extremely effective *in vivo* method for non-invasive measurement of concentration and visualization of spatial distribution of collagen type I in papillary and reticular dermis.

Series of works described the results of TPM application for histopathological analysis of human skin biopsy samples with the main aim to identify cancerous tumors [38–41]. In particular, it was shown that detection of extracellular matrix changes using TPM allows for monitoring metastasis and cancer progression [42].

Anker et al. [43] showed that AF and SHG signals analysis allows to evaluate the structural changes of epidermis related to keratinization disorder. SHG signal analysis has been also used for clinical studies of differences between healthy and mechanically damaged skin [44, 45]. In particular, it was shown that skin elasticity depends on isotropic properties of collagen. At the same time, for keloid tissues and scars, collagen fibers are straighten [46–48], which results in loss of skin elasticity. Decreased skin elasticity and alteration of collagen type I have recently been observed in tattooed skin [49]. A similar effect, as well as a decrease in the thickness of collagen fibers, was observed in the skin after transplantation [50]. Transdermal injection of collagen is actively used to increase the efficiency of skin scars and wounds healing. Ma et al. [51] suggested a method based on TPM use allowing for *in vivo* assessment of transdermal absorption of collagen in real time. Various methods are used to assess changes in collagen type I morphology. In particular, methods based on fast Fourier transform [52–56], Hilbert transform [57], as well as texture analysis [58] are widely used for SHG signal analysis. Kröger et al. [59] proposed a set of parameters for the methods described above with the main aim to *in vivo* classify different morphological structures of collagen type I in skin. Also the so-called “collagen index” defined as signal intensity ratio $(\text{SHG}-\text{AF})/(\text{SHG}+\text{AF})$ was implemented to determine the concentration distribution of collagen type I in skin [32, 60]. This method is often used by dermatologist for *in vivo* estimation of collagen type I concentration [32, 60–63].

In addition to the standard TPM, polarization-sensitive SHG microscopy approach is also widely used

for collagen assessment in skin [64]. For this method SHG signal is registered at different orientations of linear polarization of laser source. After, the degree of depolarization in the registered signal is evaluated. A number of works showed high efficiency of this method for detection of various skin diseases [65, 66], as well as for monitoring of structural changes related to skin aging processes [67, 68].

4 Mechanisms of Optical Clearing

The strong light scattering of the skin significantly limits the applicability of the methods described above only to the upper layers of the skin, since the probing depth for TPM and MPT methods in skin is $\approx 150\text{--}200\text{ }\mu\text{m}$. OC of skin allows to significantly increase the probing depth of TPM, as well as to improve the registered image quality by increasing the intensity of recorded SHG and AF signals. The most used OC method for skin imaging is local application of immersion OCAs. Currently, three OC mechanisms are generally accepted [69, 70]. The first mechanism is the refractive index matching of the skin components due to the penetration of OCA into tissue [69–73]. The second mechanism is related to the osmotic action of the OCA, resulting in dehydration of the biological tissue due to high hyperosmolarity [71–75]. The third mechanism is related to the reversible dissociation of collagen fibers after OCAs application and subsequent changes in collagen structure organization [74–76], which in turn considerably reduces light scattering caused by collagen structures [76, 77]. All three described mechanisms of OC acts simultaneously during tissue treatment with OCA. Nevertheless, the relative contribution of each mechanism is usually unequal and depends on the properties of the OCA and the biological tissue. Series of works showed that dehydration of the skin caused by the OCA application leads to scattering reduction due to the displacement of water from the areas between the collagen fibers. This process also contributes to the refractive index matching process between different skin components [71, 78]. In particular, the refractive index for human epidermis is 1.44 at 590 nm wavelength. For the same wavelength, the refractive index for dermis is 1.39, for water is 1.33, and, for example, for 70% glycerol solution in water is 1.43 [79, 80].

The OC efficiency depends on a number of factors, e.g. concentration of OCA and its refractive index, its osmolarity, treatment time, as well as the skin optical properties, its permeability to the molecules of a particular OCA, etc. In most works, OC efficiency is considered as the ratio between the intensity of signal recorded after OCA application and intensity of signal in case of absence of OCA treatment. In this way, values > 1 characterize OC efficiency according to the principle: the higher the value, the better the OC efficiency. In case of *in vivo* application, OCA effectiveness also depends on the physiological parameters of the skin, particularly on its temperature, blood saturation, metabolic response to OCA, etc. [81, 82]. Additionally, chemical enhancers such as DMSO, linoleic and oleic acids, ethanol,

propylene glycol, etc., are widely used to increase OCA efficiency and improve OCA permeability into biological objects. [13, 83, 84]. Moreover, DMSO, propylene glycol, and oleic acid themselves can be considered as OCAs [82, 85–87]. In addition to the use of chemical enhancers, the various physical methods of skin surface pre-treatment (e.g. epidermis micro-damaging, removal of part of the stratum corneum by applying tape stripping procedure, microdermabrasion, photothermal and mechanical perforation, laser irradiation of the skin surface, electrophoresis, ultrasound, degreasing, etc.) are also widely used to improve OC efficiency [88–91].

5 Influence of the Optical Clearing on the SHG Signal Intensity

Work [92] presents results of investigation of 100% glycerol topical application on rodent tail tendon and skin dermis samples. It was shown that the use of glycerol resulted in 100-fold increase in skin optical transmittance for 400 to 700 nm wavelengths. At the same time, application of OCA resulted in a 3-fold decrease in SHG signal. This effect was reversible, and SHG signal intensity returned to the initial values after rehydration of the samples by placing them in physiological solution. The authors suggested that decrease in SHG signal was caused by the reversible dissociation and disturbance of collagen fibers organization in response to OCA application. The change in collagen fibers organization also leads to a decrease in optical transmittance, which is contradictory. A more reasonable explanation of this effect was given in work [93], where SHG polarimetry was implemented to study changes in SHG signal from chicken skin dermis before and after dehydration. It was shown that dehydration of the skin resulted in decrease in SHG signal intensity by $\approx 75\%$. Thus, it was suggested that the decrease in SHG signal intensity is related not to the efficiency of SHG signal generation in the skin, but mainly to the change of its linear optical properties, namely the scattering coefficient. Also, the possible reason of 3-fold decrease in SHG signal can be due to the fact that the volumetric density of the skin increases with dehydration, resulting in the increase of the linear absorption coefficient. At the same time the linear scattering coefficient may stay unchanged due to immersion effect of OCA. Further study of skin samples fixed in formalin showed that their SHG-polarization diagrams practically did not change. At the same time, the SHG signal increased. In this case tissue fixation resulted in formation of cross-links in collagen. Thus, it was suggested that cross-links formation does not influence the collagen fibers orientation but influences SHG signal intensity. In work [94] it was shown that application of sucrose (67.1%) and polyethylene glycol solution resulted in 2-fold decrease in SHG signal intensity from mouse skin. Moreover, reversible change in skin polarization and collagen fibers distribution after OCA application was observed.

Hovhannisyan et al. [95] demonstrated the influence of application of glycerol aqueous solutions with

different concentrations on SHG signals from the chicken tendons and skin. Authors showed that TPM method allows to separate in time the different processes occurring after OCA application to the skin. It was shown that during the tissue treatment with OCA, the primary

process was a rapid dehydration of the skin with accompanying contraction of collagen fibers. This was followed by the process of relatively slow penetration of OCA into the interfibrillar space of collagen.

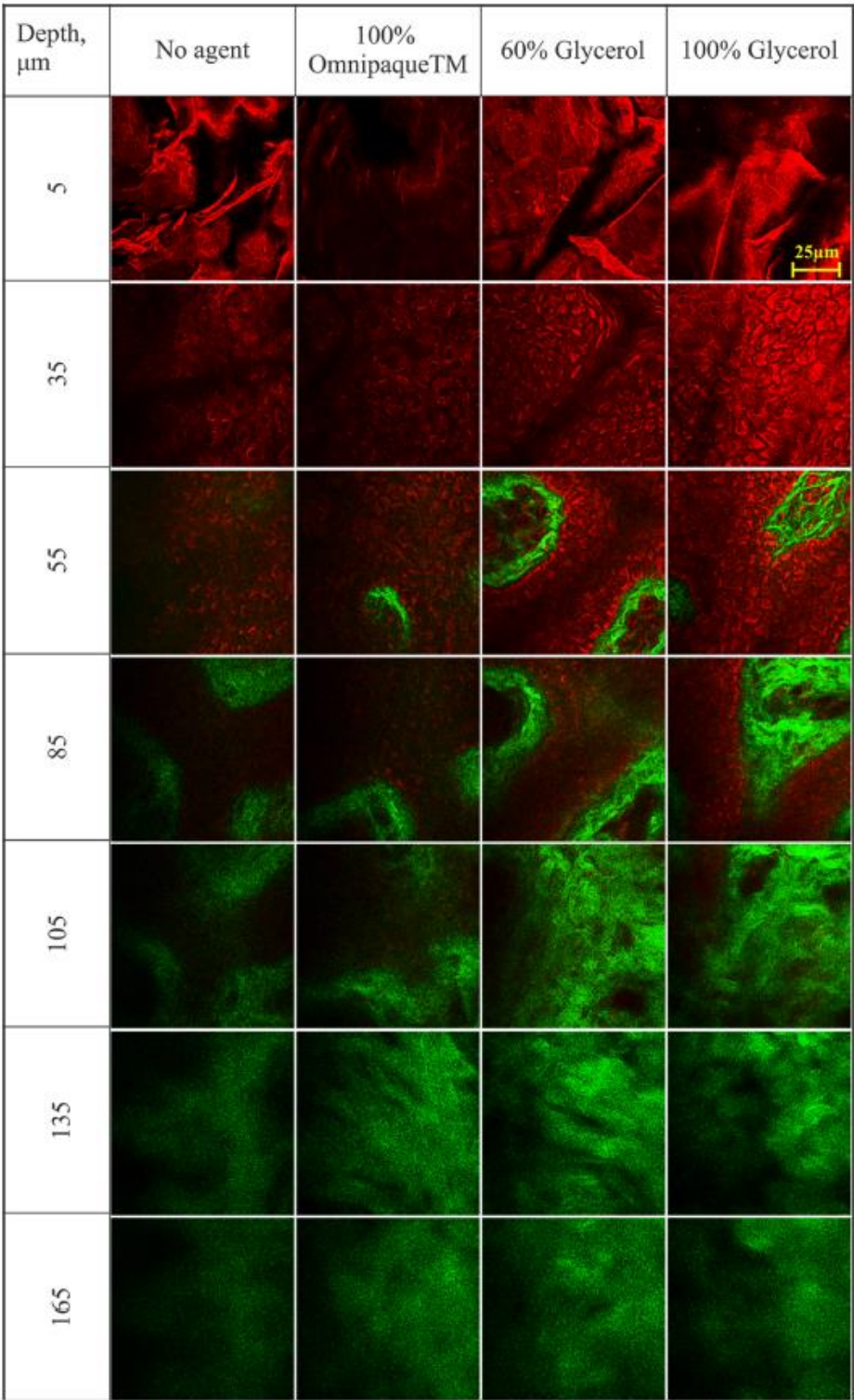


Fig. 2 Structural images for different porcine skin layers obtained *ex vivo* using TPM after Omnipaque and glycerol solutions applications, as well as for a control sample without OCA Red color corresponds to AF signal. Green color corresponds to SHG signal. Reprinted under CC BY 4.0 license from Ref. [79].

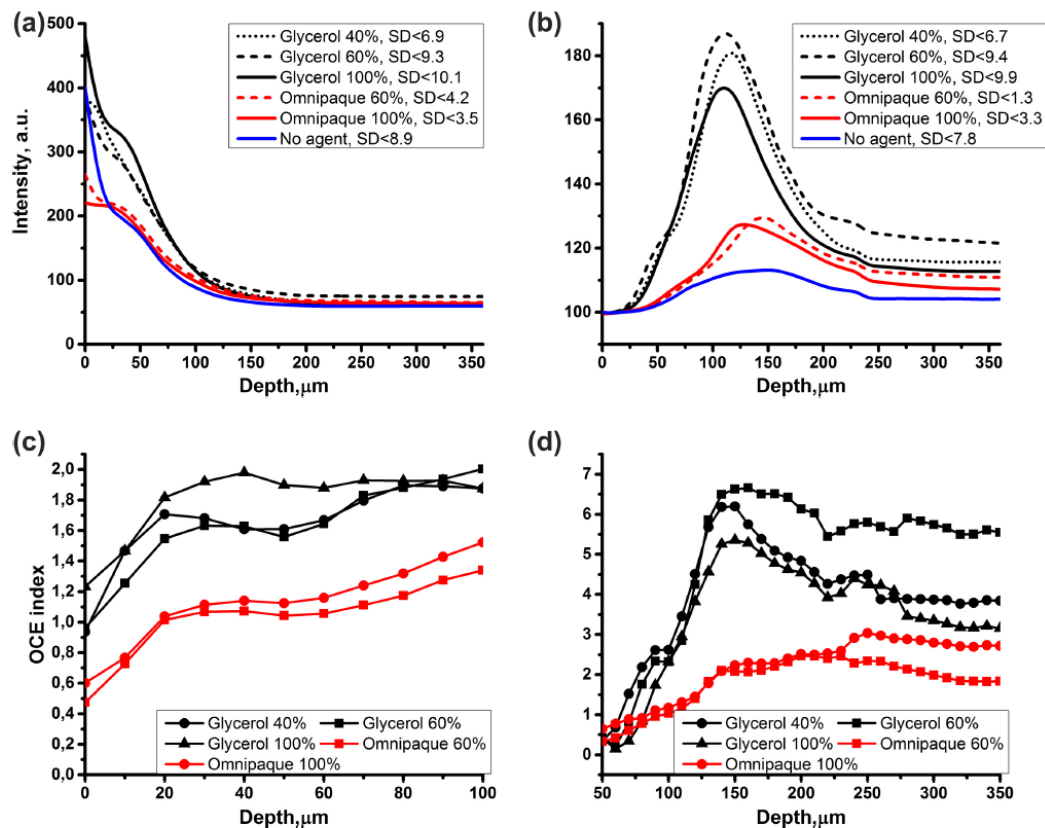


Fig. 3 Averaged in-depth AF (a) and SHG (b) signal intensity distributions obtained *ex vivo* using TPM after Omnipaque and glycerol solutions applications, as well as for a control sample without OCA. SD is the standard deviation for AF and SHG signals. The OC efficiency for AF (c) and SHG signals (d) after Omnipaque and glycerol solutions applications, as well as for a control sample without OCA. Reprinted under CC BY 4.0 license as is from Ref. [79].

It was demonstrated that application of higher concentrations of glycerol increased OCA efficiency and SHG signal intensity. In particular, 100% glycerol led to 4.75-fold enhancement in SHG signal, whereas a mixture of glycerol and phosphate buffer solution (50%/50%) led only to 1.7-fold enhancement. This effect may be related to the fact that when OCA penetrates into the skin, the skin water molecules are partially replaced by OCA molecules, leading to a decrease in refractive index values gradient between the different skin components. Thus, application of higher concentrations of glycerol has a greater OC effect due to a more pronounced replacement of skin water molecules by OCA molecules and the proximity of the refraction index values of glycerol (≈ 1.43) and skin components (for epidermis ≈ 1.44 , for dermis ≈ 1.39) comparing to water (≈ 1.33). Authors demonstrated that use of OC allowed to increase the depth of SHG signal registration from 50 μm to 500 μm . It was also shown that the use of both 100% glycerol and a mixture of glycerol and phosphate buffer solution had reversible effects.

Wen et al. [96] showed that 30 min application of 75% glycerol solution to the rat skin reduced both tissue scattering and reflection coefficients. It was also noted that the thickness of the dermis during this time interval decreased by 1.13 times due to strong skin dehydration. Also, SHG signal was significantly reduced due to

dissociation of collagen fibers. Nevertheless, for 10-min exposure by 20%-, 30%-, and 75%- glycerol solutions, SHG signals were higher comparing to the control skin samples without OCA treatment. Despite the collagen fibers compression (decrease of collagen fibers diameter up to 1.38 times), they did not dissociate and did not break off, which allowed to assume that one of the OC mechanisms relates to decrease of biological object layer thickness and subsequent more dense and orderly packing of collagen fibers.

Cicchi et al. [97] demonstrated the effects of different OCAs application on increment of the probing depth for SHG measurements, as well as on increment of contrast for obtained images. In particular, it was shown that the use of glycerol for 7 min allowed to increase the depth of SHG signal registration from 40 to 80 μm . Moreover, the contrast of the images increased thousands of times at a depth of 60–80 μm . The use of glucose and propylene glycol also resulted in a comparable increase in contrast. Authors showed that the contrast enhancement depends on OCA application time. It should be noted that stratum corneum provides the skin barrier function [98, 99] and prevents penetration of substances into the living epidermis and dermis. It has been shown that propylene glycol does not penetrate through the stratum corneum in a relatively short time [100], so the complete or partial

removal of the stratum corneum is implemented in practice to enhance OC [79, 90].

OCA application allows to reduce the tissue scattering and increase the further fluorescence yield until the OC front reaches the depth of the emitting fluorophores. In this case, the total path length of the photon that excites the fluorescence is significantly reduced. Thus, the probability of fluorescence excitation is reduced. On the other hand, with reduced scattering, the probability that the fluorescence photon still reaches the detector increases. On this basis, it should be kept in mind that a strong OC, e.g., due to prolonged exposure by OCA, can lead not only to an increase but also to a loss of the useful signal [101]. Particularly, it was shown in Ref. [100] that a 3-h *ex vivo* human skin treatment with DMSO solution leads to ≈ 1.4 -fold decrease in the SHG signal intensity.

Sdobnov et al. [79] investigated effects of application of OCAs with different osmolarity on SHG and AF signals from porcine skin *ex vivo*. Topical application of iohexol (OmnipaqueTM(300)) and glycerol solutions with different concentrations within 1 h allowed to increase the probing depth in skin from 200 to 350 μm . It was shown that 100% glycerol had a lower OC efficiency for SHG signal comparing to aqueous solutions with lower concentrations. Particularly, 100% glycerol application increased SHG signal up to 5.4 times, whereas 40% and 60% glycerol solutions application increased the signal up to 6 and 6.6 times, respectively. This effect may be related to the high viscosity of 100% glycerol preventing OCA penetration into the skin. At the same time, in case of AF signal, the use of 100% glycerol resulted in higher OC efficiency comparing to 40% and 60% solutions (increase in AF signal up to 2, 1.8, and 1.8 times, respectively). Also, aqueous glycerol solutions showed higher OC efficiency comparing to aqueous iohexol solutions for both AF and SHG signals. In

particular, AF and SHG signals were enhanced only 1.5- and 3-fold, respectively, after application of 100% iohexol solution. Possibly, higher enhancement in AF signal in case of glycerol application is achieved due to strong skin dehydration. At the same time, the use of glycerol resulted in changes in the cellular structure of epidermis. Fig. 2 demonstrates structural AF and TPM images for different porcine skin layers obtained *ex vivo* after Omnipaque and glycerol solutions applications, as well as for a control sample without OCA. Fig. 3 demonstrates the corresponding data on increase in the intensity of AF and SHG signals, as well as the efficiency of OC for different OCAs. It is also important to note that in case of iohexol solutions application AF signal at small depths (up to 50 μm) decreased in comparison with control skin samples and samples treated with glycerol solutions. The possible reason can be the fact that iodine contained in iohexol can decrease the fluorescence signal during its penetration into a biological sample. In particular, it was shown in Ref. [102] that interaction between iodine and fluorophores leads to dynamic quenching of fluorescence.

In work [103] the effect of 100% and 50% glycerol solutions application on SHG signal intensity and third harmonic generation (THG) signal intensity from the human skin was investigated *ex vivo* (see Fig. 4). It was shown that 100% glycerol application resulted in 1.15-fold reduction of THG signal for stratum corneum and epidermis. For the dermis, the THG signal was slightly increased after OCA application. At the same time, in case of SHG signal, application of OCA resulted in 2-fold enhancement in the registered signal from dermis. Application of 50% glycerol solution resulted in 1.3-fold enhancement of THG signal from stratum corneum and epidermis. Intensity of SHG signal also increased significantly.

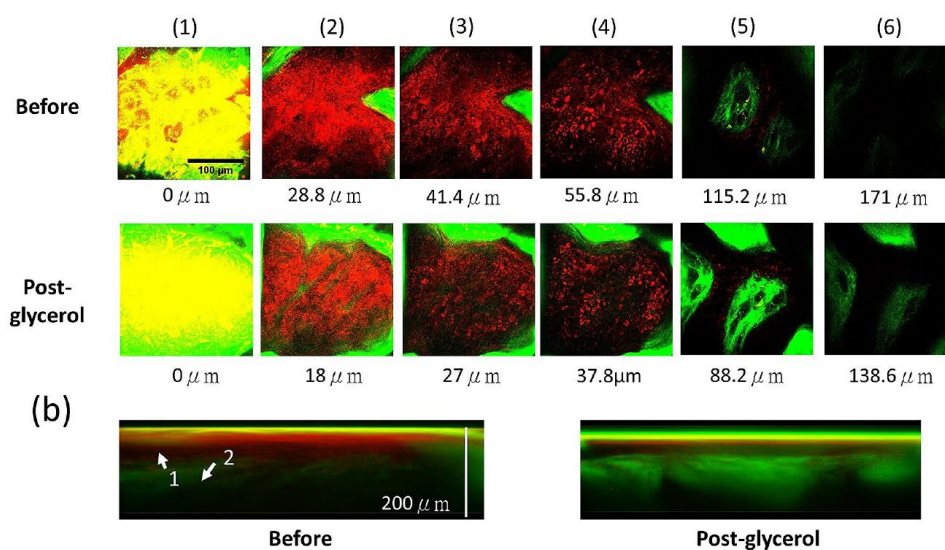


Fig. 4 (a) Structural images for different layers of human skin obtained *ex vivo* before and after treatment with 100% glycerol. Red color corresponds to the THG signal. Green color corresponds to the SHG signal. (b) Cross-sectional images of human skin samples obtained *ex vivo* before and after treatment with 100% glycerol. Reprinted under CC BY 4.0 license from Ref. [103].

Authors suggested that the use of 50% glycerol solution provides the compromise between the reduction of scattering and refractive indices matching between the skin components, leading to increase in signal intensity. It has also been suggested that the key role in skin OC is the interaction of OCA directly with the stratum corneum. In particular, stratum corneum shrinkage and refractive index matching due to OCA penetration contributed to increase in SHG signal intensity in dermis. One of the OC methods is mechanical compression of biological objects [104, 105], which is actually an analogue of skin shrinkage as a result of dehydration caused by the hyperosmotic action of OCAs. Also, the described result agrees well with other studies where it has been shown that glycerol concentrations in the range 40–60% are preferable for OC due to the low viscosity comparing to the pure glycerol (14-fold difference in viscosity), as well as due to lower decrease in the refractive index caused by dilution with water [106]. Also, the ratio between the water content in OCA and skin is important parameter. In particular, in stratum corneum and living epidermis the water concentration averages are 45% [107] and 60% [91, 107], respectively. In this case, water practically does not participate in diffusion processes and mainly glycerol molecules diffuse into the skin [108]. Each glycerol molecule binds about six water molecules [109] reducing the light scattering of superficial skin layers and leading to refractive index matching between the skin components. This theory agrees well with the results described above [79, 103], as well as with the results presented in works [106, 110, 111].

In addition to the skin, OC influence on SHG signal is actively studied for the other biological objects. For example, Genin et al. [112] showed that application of 50% aqueous glycerol solution on rodent tendons and striated muscles allowed to obtain information on SHG signal polarization response at the depths up to 200 μm due to reduction of light scattering in upper tissue layers. In Ref. [113] it was shown that OC with fructose solutions allowed to achieve 5-fold increase in probing depth for human cartilage. Zhang et al. [114] presented OC protocol allowing to achieve reversible temporal OC of a mouse skull. Also, the proposed method allowed to significantly increase both the probing depth (from 120 to 300 μm) and SHG signal intensity (more than 10-fold) during transcranial blood flow imaging in the mouse brain. In Ref. [115] it was shown that application of

glycerol, DMSO and buffer solution allows to increase the depth of SHG signal detection in the anterior leaflet of sheep mitral valve up to 800 μm comparing to 100 μm in case of control samples without OC. At the same time, application of OCA led to strong dehydration of biological objects and conformational changes in collagen.

6 Conclusions

Various physical and chemical methods of skin pre-treatment are widely used to enhance the probing depth and image quality of skin TPM. The most effective and promising method is immersion OC and/or its combined use with the discussed optical methods. This article presents a brief review of works devoted mainly to the study of OC influence on SHG and AF signals from skin. The theory of two-photon excitation is given, and the basic principles and mechanisms of OC are described.

Despite the disagreement in interpretation of the experimental data, and particularly in the evaluation of contribution of different OC mechanisms to its overall efficiency, all the studies presented in this paper showed that the effect of OCA application on skin allows to increase the probing depth. In addition, OC method improves the overall quality of detected images due to a better focusing of laser radiation into the skin caused by reduced scattering. Also, OCA application leads up to 6.6-fold increase in intensity of skin SHG signal and up to 1.8-fold increase in intensity of skin AF signal by tissue dehydration, collagen dissociation and refractive index matching mechanisms. Thus, OC method is extremely promising for further *in vivo* and *ex vivo* use on mammalian skin and, in particular, for investigation of structural changes of collagen type I in the dermis at the depths exceeding 150 μm .

Disclosures

The authors declare no conflict of interest.

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