

Optical Clearing for Deep Skin Imaging with Optical Methods

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Abstract. Skin imaging with optical methods is always limited in depth mainly due to the strong scattering of the excitation and emission light, which significantly reduces the contrast of the images and the probing depth. One of the reasons for the scattering is the mismatch in refractive indices between the skin components, which can be minimized using an optical clearing procedure – the topical application of immersion liquid on the skin. Professor Valery V. Tuchin is a well-known scientist who plays a major role in the field of determination and maintenance of the optical properties of biological objects, including skin, and was a driving force in the development of the optical clearing method. In addition, Professor Valery V. Tuchin can be considered the “Father of Biophotonics in Russia” due to his long-standing, highly effective and globally recognized work in the field of biophotonics, biomedical optics and laser medicine. The following article provides an overview of the work carried out at the Charité – Universitätsmedizin Berlin, Department of Dermatology, Center of Experimental and Applied Cutaneous Physiology, in cooperation with Professor Valery V. Tuchin, on the improvement of image contrast and the increase of probing depth in the skin using exemplary optical methods in combination with optical clearing. © 2024 Journal of Biomedical Photonics & Engineering.

Keywords: Raman spectroscopy; confocal Raman microspectroscopy; two-photon tomography; second harmonic generation; two-photon excited autofluorescence; epidermis; dermis; deep tissue imaging.

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1 Introduction

Optical microscopic and spectroscopic methods are widespread in dermatology to image the hidden structures and substances in the skin. These include linear optics-based methods such as laser scanning microscopy in reflectance and fluorescence mode, confocal Raman micro spectroscopy, optical coherence tomography; and non-linear optics-based methods such as two-photon tomography, coherent anti-Stokes Raman scattering, stimulated Raman scattering – comprehensively reviewed in Refs. [1–7]. The primary limitation of all optical methods used in dermatology is their low imaging depth, which is generally limited to a few hundred μm

and considerably restricts skin diagnostics. The reason for the imaging limitation is a strong scattering of the skin – the light is reflected multiple times within the skin components with different refractive indices ($n_{\text{lipids}} = 1.47$; $n_{\text{collagen}} = 1.47$; $n_{\text{nuclei}} = 1.39$; $n_{\text{water}} = 1.33$; $n_{\text{blood}} = 1.4$; $n_{\text{air}} = 1.00$) [4, 8]. The greater the refractive index mismatch along the light propagation, the stronger the scattering.

To minimize the mismatch of reflective indices, the immersion optical clearing (OC) method was introduced by Professor Valery V. Tuchin in 1997 [9], who is a pioneer of this development and of further research on biological objects using optical methods in combination with OC [8, 10–14]. The basic idea of the OC method for

the skin is the topical application of a non-toxic optical clearing agent (OCA) with a refractive index comparable to the refractive index of the main skin components (proteins and lipids), with the ability to squeeze out interstitial fluid (mainly water) and replace it with an OCA [8, 9, 15]. The most frequently used OCAs are glycerol ($n = 1.47$ at 785 nm) [16], glycerol in water 60%/40% ($n = 1.41$ at 785 nm) [16], glucose and fructose ($n = 1.46$ – 1.48) [17], propylene glycol ($n = 1.43$) [17], iohexol ($n = 1.43$ at 760 nm) [15], polyethylene glycol ($n = 1.46$ at 930 nm) [18], their mixtures and solutions in water. Topical application of OCA minimizes the mismatch of the refractive indices considerably, reduces the scattering and thus increases the probing depth. However, the OC method has a few limitations when used *in vivo* – not every OCA is biocompatible with the skin and can induce irreversible changes to skin components at the micro level, so the concentration used and the application time should be considered [17].

Since many scientific groups worldwide are following the development of OC and the successful combination of optical methods with OC has been demonstrated for decades [10, 14, 17, 19–22], we summarize below the results in this field achieved in the past by the authors of this manuscript, previously employed at the Center of Experimental and Applied Cutaneous Physiology, Department of Dermatology, Charité – Universitätsmedizin Berlin, in cooperation with Professor Valery V. Tuchin. These results include application of OC using confocal Raman micro spectroscopy and two-photon tomography in dermatological research.

2 Results and Discussion

2.1 Optical Clearing of the Skin – Important Application Features

The stratum corneum (SC), the outermost layer of the epidermis, consists of denucleated cells (corneocytes) embedded in a structurally organized lipid matrix, is hardly permeable and maintains the skin barrier function [23–25]. Consequently, the topically applied OCA is mainly stored in the superficial SC depth and does not penetrate the deeper skin layers. To realize the OC method efficiently, OCA should permeate the SC. Therefore, in most studies, OCA penetration is facilitated by removing part or all of the SC by means of tape stripping, creation of micropores, micro ablation, etc. [8]. Another option is to mix a target OCA with penetration enhancers, such as ethanol, dimethyl sulfoxide, thiazone, linoleic and oleic acids [8]. Thus, OC is always associated with the influence on the SC with its complete or partial destruction – it is classified as a low-invasive method. *In vivo* applications are not too common compared to the widely used *ex vivo* investigations to improve probing depth by imaging methods [18, 19, 26–31]. For *in vivo* OC, mixtures of glycerol/dimethyl sulfoxide or propylene glycol/water with a proportion of $\approx 70/(5\text{--}20)/(10\text{--}25)\%$ are popular, but not limited to these OCAs. *In vivo* skin OC has its action

time – it reaches its maximum 10–15 min after treatment, the effect lasts about 45 min and then slowly decreases due to the substitution of OCA by interstitial fluid.

2.2. Confocal Raman Micro Spectroscopy (CRM)

CRM is a non-invasive spectroscopic method that provides depth-resolved information about skin composition with chemical sensitivity down to a depth of $\approx 50\text{ }\mu\text{m}$ [32, 33]. CRM is very popular in skin research and is used to study structural and physiological features of the skin *in vivo* and *ex vivo* [32, 34, 35]. CRM helps to determine penetration depth of topically applied substances [32, 36, 37] and to evaluate their influence on the skin [38, 39], to identify changes in the SC between healthy and non-malignant diseased skin [40–43], and to determine cancerous tissue [44–50].

The enhanced probing depth by combining CRM and OC methods [51–53] was confirmed by Sdobnov et al. [54] using porcine ear skin with destroyed SC *ex vivo* and 70/30% glycerol/water and 100% iohexol as OCAs. The results show a significant increase in Raman band intensities (increment factor >1) 30–60 min after topical treatment of the skin with glycerol (for 40–240 μm depth) and iohexol (for 160–240 μm depth) compared to untreated skin. Superficial skin areas show no OC efficiency (increment factor <1), which is due to the presence of OCA in these skin depths. The authors demonstrated that OCAs penetrate into the dermis, provide an OC and cause a significant dehydration of the collagen, which is more pronounced after application of glycerol than iohexol. Comparable results were obtained by Jaafar et al. for porcine dura mater *ex vivo* (glycerol/water solution in different proportions as OCA) [55] and for porcine lung tissue *ex vivo* (e-cigarette liquid as OCA) [56] using a combination of CRM with OC.

In addition, Yanina et al. [16] investigated OC on intact porcine skin *ex vivo* without disturbing the SC by applying a mixture of glycerol and dimethyl sulfoxide and found that a solution of 80/20% glycerol/dimethyl sulfoxide for 15–45 min was optimal to permeate the SC and reach the viable epidermis.

It is known that the contact of OCA with a biological object leads to its dehydration, but the question of the changes in water mobility states after OCA treatment was open. Based on the decomposition of Raman spectra and determination of water mobility states, Sdobnov et al. [57, 58] studied the interaction of 70/30% glycerol/water and 100% iohexol on water homeostasis in porcine skin with the removed SC *ex vivo*. Fig. 1 shows that glycerol induced the highest water displacement from the epidermis and dermis (depths 0–200 μm) and a decrease in the concentrations of all water mobility states (tightly-, strongly-, weakly-bound, and unbound water), while the hydrogen bonding state of the water (ratio of weakly bound to strongly bound water) did not change significantly (0–200 μm) after the OCA treatment. It is concluded that the weakly bound and strongly bound

water mobility states, which are most prevalent in the skin, are preferentially involved in OCA-induced water flux in the skin, and are thus mainly responsible for OC efficiency [57]. Treatment with both OCAs leads to a significant decrease in the fluorescence intensity of the Raman spectra at a depth of 0–50 μm , which is explained by the presence of the OCAs in this skin region due to penetration [15, 58].

The main limitation of using a combination of CRM and OC is the strong overlapping of skin- and OCA-related Raman bands in a broad spectral range, which hinders the calculation of some structural and physiological features of the skin. Another aspect is an *in vivo* application – here the safety of the applied OCA and the doses should be confirmed.

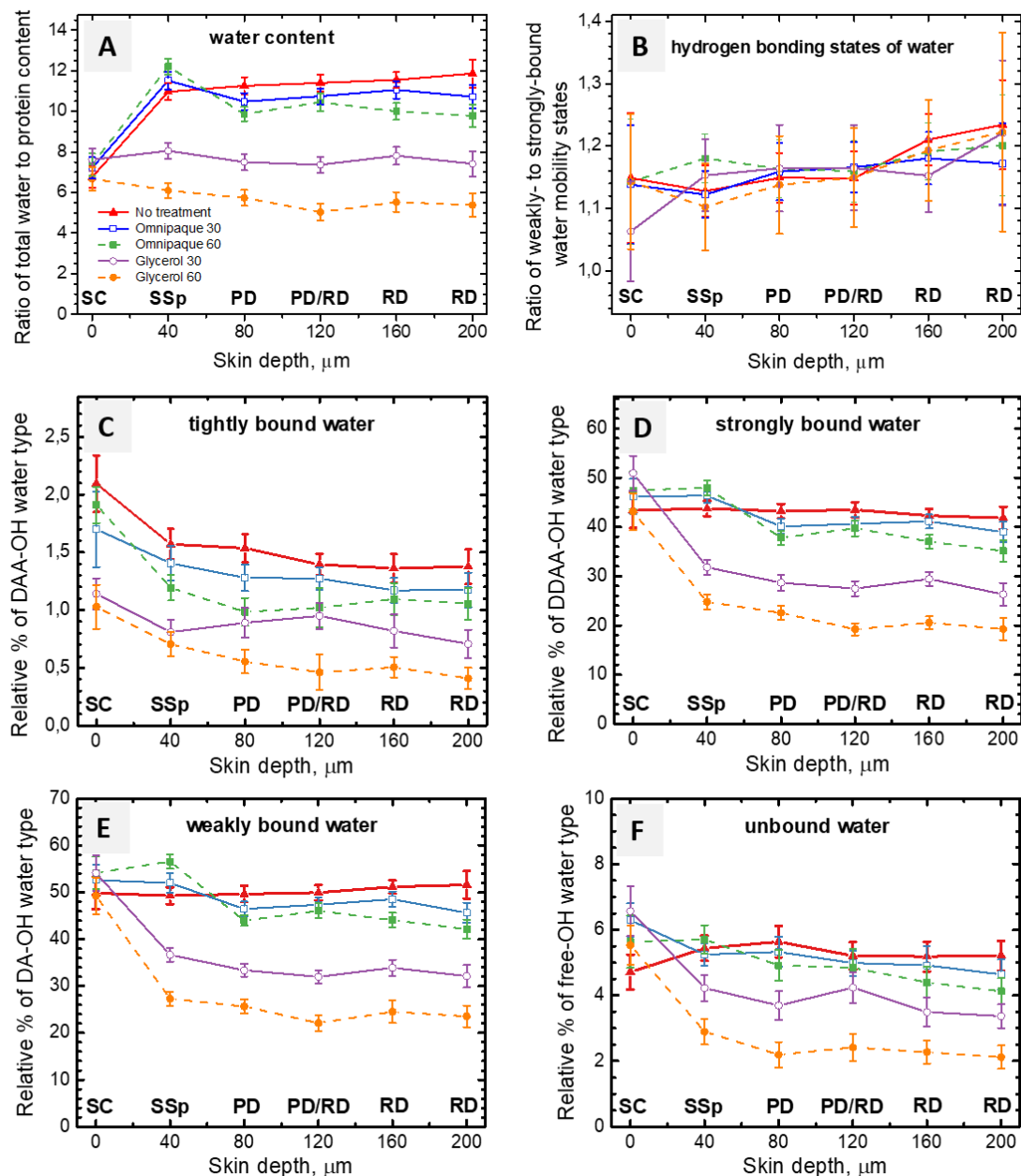


Fig 1. Skin depth profiles of (A) total water content (determined by the ratio of total water to proteins content), (B) hydrogen bonding state of water molecules (determined by the ratio of weakly bound to strongly bound water), (C) concentration of tightly bound water, (D) concentration of strongly bound water, (E) concentration of weakly bound water, and (F) concentration of unbound water determined on porcine ear skin *ex vivo* using CRM (excitation at 671 nm, detection at 3000–3750 cm^{-1}). Thick red line – untreated skin; blue solid line/green dotted line – skin treated with Iohexol (Omnipaque) for 30/60 min; purple solid line/orange dotted line – skin treated with glycerol for 30/60 min. SC – stratum corneum; SSp – stratum spinosum; PD – papillary dermis, RD – reticular dermis. Reproduced with permission from Ref. [57].

2.3. Two-Photon Tomography (TPT)

TPT is a non-invasive imaging method that enables depth-resolved visualization of skin structures by measuring two-photon excited autofluorescence, fluorescence lifetime imaging (TPE-FLIM) and second harmonic generation (SHG) to a depth of $\approx 150\text{ }\mu\text{m}$. TPT is used in skin research with a focus on the visualization of epidermal and dermal structures including cells of different types [59–62], tattoos [63], collagen and elastin in the dermis [64–66], for the determination of the penetration of topically applied substances in the skin [2, 67, 68], and in clinical research (mainly in skin cancer diagnostics) [69–72] both *in vivo* and *ex vivo*. Compared to methods based on linear optics, TPT offers excellent subcellular axial ($<2\text{ }\mu\text{m}$) and lateral ($<0.6\text{ }\mu\text{m}$) resolution and the highest imaging depth. Despite the high resolution, the images in the dermis at a depth exceeding $80\text{ }\mu\text{m}$ are partially blurred [60, 61, 63] and can still be improved. The selectivity of TPT to different

substances (measured with SHG and TPE-FLIM) is unique in *in vivo* skin imaging.

Skin scattering reduces image quality and probing depth, so the combination of TPT with OC may be useful to minimize these limitations. Cicchi et al. [73] were the first to show that the OCAs glycerol, propylene glycol and glucose at high concentrations effectively improve image contrast and probing depth in TPT of human skin *ex vivo*. Sdobnov et al. [74] investigated porcine skin *ex vivo* and found up to a 2-fold enhancement in autofluorescence intensity at $30\text{--}100\text{ }\mu\text{m}$ and up to a 7-fold enhancement in second harmonic generation intensity at $130\text{--}180\text{ }\mu\text{m}$ after using 40–100% glycerol in water solution. The use of iohexol also leads to an enhancement in signal intensity, but to a much lesser extent than with glycerol [15, 74, 75]. Fig. 2 demonstrates structural horizontal TPT images of porcine ear skin *ex vivo* (autofluorescence/SHG are shown in red/green) for various depths (5– $165\text{ }\mu\text{m}$) after application of 100% iohexol (Omnipaque), glycerol in water solutions, as well as for an untreated skin sample.

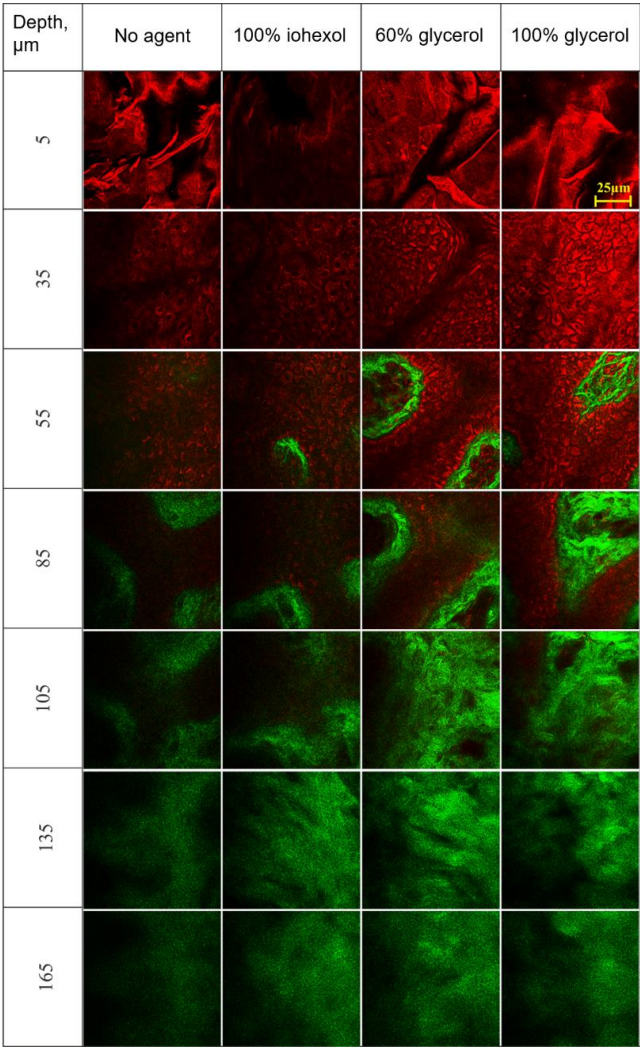


Fig. 2 Exemplary TPT images of different skin layers obtained *ex vivo* for porcine ear skin samples using different iohexol (Omnipaque) and glycerol solutions as OCAs. The red colour corresponds to two-photon-excited autofluorescence and the green colour corresponds to SHG intensities. The image size is $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$. Reproduced with permission from Ref. [74].

These results show that both glycerol and iohexol solutions increase the probing depth and image quality in the skin so that the deep-located regions can be examined. Glycerol is more effective than iohexol at the concentration and application time values used. However, since iohexol (Omnipaque) is a radiocontrast agent used *in vivo* in X-ray microscopy and an approved pharmaceutical agent for topical or systemic use with no known impact on the skin, it is more promising than glycerol for *in vivo* application.

One of the future prospects for reducing the influence of scattering on image quality and probing depth may be the combination of OC with a TPT device upgraded with adaptive optics elements, in particular spatial light modulators, used for wavefront correction [76–78], similar to what Yu et al. [79] showed for optical coherence tomography.

3 Conclusions

The effect of OC is generally observed in all biological objects investigated using optical methods and is here

presented for CRM and TPT used for skin diagnostics. The improvement in image quality and probing depth due to a reduced scattering using optical methods is clearly pronounced after OC and is consistent with the results presented by other authors. The effectiveness of OC depends on the type of OCA, its concentration, and the application time. Although many OCAs are already used *in vivo*, an important limitation of OC method is still its application *in vivo*, as not all OCAs are biocompatible with the skin and can potentially cause irreversible changes in skin components. Therefore, future studies should be considered for the optimization of the composition and concentration of OCAs with the aim of achieving fast OC effect and rapid recovery with no or minimal effects on skin components.

Disclosures

The authors declare no conflict of interest.

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