Enhanced optical clearing of skin in vivo and optical coherence tomography in-depth imaging

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1 Introduction

For a strong light-scattering tissue like skin, ballistic and quasi-ballistic photons of incident light cannot penetrate deeply into the tissue, which limits the ability of many optical imaging methods to image deeply. Imaging methods such as optical coherence tomography (OCT), confocal reflectance/fluorescence microscopy, second-harmonic generation microscopy, and 2-photon microscopy are limited by the optical scattering properties of the skin to superficial depths. Optical clearing using high refractive index and hyperosmolarity agents can reduce the scattering of biological tissues. With this approach, better optical imaging depth and contrast have been presented and deeper optical treatment has been achieved.

An effective way to achieve optical clearing in vitro experiments is to immerse an excised skin sample in an optical clearing agent (OCA) with high refractive index and hyperosmolarity. By immersion, the OCA directly interacts with the dermis, inducing refractive index matching, dehydration, collagen fiber dissociation, or anisotropy factor increase, which all contribute to reducing the effective scattering in skin. However, non-invasive optical clearing of skin in vivo is more difficult. The outermost layer of the skin, the stratum corneum, presents a significant barrier to topically applied OCAs and is hence responsible for the poor optical clearing effect. To break the barrier of the stratum corneum, multiple penetration enhancing methods have been introduced, such as chemical enhancers, ultrasound, microneedles, flashlamp, laser fractional ablation, and photo-irradiation. Also, physical massage is commonly known to enhance penetration of topical agents into skin.

Recently, we reported imaging dermal blood flow on rat dorsal skin by optical clearing using PEG-400 with the chemical penetration enhancers thiazone and 1,2-propanediol. Optical coherence tomography (OCT) has the ability to image tissues in different depth in vivo. This ability makes OCT a powerful tool to monitor the optical clearing process in tissues.

In this paper, OCT imaging was applied to monitor the optical clearing of rat skin in vivo. The optical clearing efficacy was assessed quantitatively by analyzing the OCT reflectance from different depths. The synergistic effect of an OCA (PEG-400), two chemical penetration enhancers (thiazone and 1,2-propanediol), and physical massage on optical clearing was tested.

2 Materials and Methods

A commercial OCT system (model OCP930SR, Thorlabs, Newton, NJ, USA) working at 930 ± 5 nm with 100 ± 5 nm FWHM, an optical power of 2 mW, a focal length of 20 mm, a maximum image depth of 1.6 mm, a lateral resolution of 20 μm, and an axial resolution of 6.2 μm in air was used in this investigation. The image depth did not exceed 1 mm. The sensitivity drop-off versus depth of the SD OCT system was modest over this 1 mm range, and was ignored.

An OCA, PEG-400 (refractive index: 1.47, Tianjin Kermel Chemical Reagent Co., Ltd. Tianjing, China), and two kinds of chemical penetration enhancers, thiazone [[benzisothiazol-3(2H)-one-2-butyl-1,1-dioxide], refractive index: 1.47, Guangzhou Heming Trading Co., Ltd. Guangzhou, China] and 1,2-propanediol (refractive index: 1.43, Tianjin Guangcheng Chemical Reagent Co., Ltd. Tianjing, China) were used in this work. PEG-400 was premixed with each chemical penetration enhancers at a PEG-400: enhancer volume ratio of 19:1.

Fifteen male 4-week-old Sprague-Dawley rats were obtained from the biological department of Saratov State University. Rats were anaesthetized with zolletil via intramuscular injection.

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The dorsal hair was shaved, and the residue hair was removed using depilatory cream. The skin was then tape-stripped 5 to 7 times with an adhesive tape to clean the skin surface and to remove the residual epilatory cream, which is important to achieve the optical clearing effect. In humans, usually more than 30 tape-strippings are required to remove stratum corneum. The rat skin stratum corneum is also several cell layers thick, and also requires many tape-strippings for removal and enhanced permeability.

The middle region of the back lateral to the backbone was chosen for OCAs treatment. Treatment sites were kept the same place for all rats. Four protocols used either PEG-400 with thiazone, PEG-400 with propanediol, PEG-400 only, or saline only. To improve the penetration of the OCAs, a soft massage was applied by moving a small plastic rod back and forth on the site of OCA application throughout the application period. A fifth protocol served as a massage-free control, with PEG-400 plus thiazone applied by using a saturated gauze without massage. Three rats were used for each of the five protocols for a total of 15 rats.

For each area of application, B-scan images were taken for the intact skin, skin after stripping, 5, 10, and 15 min after agent application. Prior to each time of imaging, the skin surfaces were wiped clean of OCAs with Kimwipes(TM) (Shanghai ANPEL Scientific Instrument Co., Ltd. Shanghai, China). OCAs were then re-applied after the imaging. The time of imaging (5 min for handling and image acquisition) was not included in the reported OCA application time. During the experiments, the anesthetized rats were fixed on a platform and the OCT probe was mounted on a rotating arm that when swung into position always measured the same skin site on the rat. Three duplicate images were acquired for each time point. Animal movements during breathing sometimes caused a fluctuation in the images, but subsequent image analysis identified the skin surface hence occasional movements were easily corrected. The acquisition time for each frame was 125 ms, with a 2-s interval between duplicated acquisitions. The mean relative standard deviation of local reflectance between three duplicated acquisitions was 7.5%.

The OCT reflectance was calibrated by using a glass-glycerol interface created by a drop of glycerol adjacent to a glass coverslip. Calibration of the OCT system was accomplished by (1) establishing the focus function of the objective lens, and (2) establishing the calibration constant, CALIB, of the system. The focus function is the sensitivity of the OCT reflectance measurement as a function of the distance of the measurement depth (z) from the focus of objective lens (zf), \( F(z - z_f) \). The skin surface defined \( z = 0 \) and was located in the upper third of the OCT imaging range. The \( F(z - z_f) \) was specified by moving a glass/air interface over the axial scanning range \( z \) of the OCT system. The CALIB was specified by measuring the OCT signal from a glass/glycerol interface at \( z = z_f \), \( M_{gg} \) [counts], which corresponded to a reflectivity of \( R_{gg} = [(1.51 - 1.47)/(1.51 + 1.47)]^2 = 1.80 \times 10^{-4} \). Then OCT measurements on skin, \( M(z) \) [counts], were adjusted to yield reflectance \( R \), where \( R = 1 \) for a mirror. Since the OCT signal is proportional to the square root of the diffuse reflectance, the reflectance was calculated as proportion to the square of OCT signal:

\[
R(z) = \left[ \frac{M(z)}{F(z - z_f)} \right]^2 \text{CALIB,} \tag{1}
\]

\[
\text{CALIB} = R_{gg} \left[ \frac{F(z - z_f)}{M_{gg}} \right]^2. \tag{2}
\]

After image acquisition, the skin surface was flattened by image analysis to enable averaging of the \( R(z) \) profile over the whole image. The flattening procedure involved finding the \( z \) position of the skin surface at each lateral \( x \) position in the image, and then translating the column of pixels at that \( x \) to bring the skin surface to a common axial position. Figure 1 shows an example of flattening the skin by image analysis. The flattening procedure is done after first correcting for the focus function of the system, i.e., correcting the axial dependence of the image intensity as a function of distance from the focus of the objective lens. The numerical aperture of the OCT system is low (<0.22, after accounting for the refraction at the air/skin surface), so the focal length of the system should not be an issue with regard to the flattening procedure. The flattening procedure will distort the tissue structure somewhat because it shifts pixels so that the surface is aligned at one constant \( z \)-axis position. But given the one-dimensionality of the skin, and because the study addresses the one-dimensional penetration of light into the skin, such spatial distortion has minimal effect on the deduced light penetration.

3 Results

Figure 2 shows the typical results for topical application of optical clearing agents by 5 different treatment protocols. A dark color represents a strong OCT reflectance and a light color represents a weaker signal due to lower reflection by the tissue in the focus and/or stronger signal attenuation to/from a particular depth \( z \). The OCT signal falls as a function of depth in the dermis. The images did not change significantly after cleaning.

![Fig. 1](https://example.com/image1.png) (a) Original OCT image and (b) the computer-flattened image of intact rat skin. Dark indicates strong reflectance, light indicates weak reflectance.
by tape-stripping with adhesive tape. With optical clearing, the upper image of the dermis becomes lighter and the boundary between the epidermis and dermis becomes less apparent. As optical clearing develops, the superficial layers of the dermis become lighter, which means less scattering is occurring, hence photons can travel deeper to image. For the groups of PEG-400 with both chemical penetration enhancers and soft massage, the most significant optical clearing can be seen, while for the PEG-400 with only soft massage there is less clearing. The effect of optical clearing can be hardly seen for the control groups of massage with saline or PEG-400 without massage.

Figure 3 is the depth reflectance, \( R(z) \), averaged over all the lateral \( x \) positions. The solid line is from the measurements of the intact skin, and the dotted line is from those after applying the mixtures with massage for 15 min. It can be found that the OCT signal from the surface is very strong, but decreases rapidly within 20 \( \mu m \). After the sharp strong signal of the surface, for the intact skin, the signal of the intact skin initially drops presumably due to the epidermis, then increases due to the superficial dermis which is more strongly scattering. The signal gradually decays versus increasing depth in the dermis. In the experimental group, the superficial signal drops due to clearing and the signal at deeper depths increases due to less attenuation by overlying tissue. The optical clearing reduces the reflectance from the upper dermis layer, e.g., 20 to 100 \( \mu m \), and enhances the signal from deeper dermis layer, e.g., 100 to 450 \( \mu m \). To illustrate the increased penetration, a threshold signal of \( 10^{-5} \) reflectance was chosen and the depth at which the signal dropped below this threshold was noted as \( Z_{\text{threshold}} \). After the 15-min application of OCAs with massage-assisted penetration, \( Z_{\text{threshold}} \) increased from 0.219 to 0.275 mm (26% increase) for PEG-400 with thiazone. The \( Z_{\text{threshold}} \) increased from 0.197 to 0.262 mm (33% increase) for PEG-400 with 1,2-propanediol.

Figure 4 shows the time-resolved reflectance from a 300-\( \mu m \) depth during five different treatment protocols. The SD bars shows the standard deviation for nine experiment repetitions (three repetitions for each site and three rats for each protocol). The two control groups, saline + massage [Fig. 4(a)] and

**Fig. 2** Computer-flattened optical coherence tomography (OCT) images of rat dorsal skin during topical application of different optical clearing agents.

**Fig. 3** The depth reflectance before and after the topical application of optical clearing agents, showing the average of three duplicate images. (a) Using thiazone as the enhancer. (b) Using propanediol as the enhancer.
PEG-400 + thiazone but no massage [Fig. 4(b)], showed no change in reflectance. The reflectance from the 300-μm depth increased ~50% with PEG-400 solution and massage but no enhancer after 15 min [Fig. 4(c)]. For PEG-400 with thiazone [Fig. 4(c)] or 1,2-propanediol [Fig. 4(d)], the 300-μm reflectance increased about 3-fold from 2 × 10⁻⁵ to 6 × 10⁻⁵ after 15 min of topical application of agents with soft massage.

Figure 5 shows bar graphs of the average $Z_{\text{threshold}}$ i.e., depth at which reflectance drops to 10⁻⁵, during five different treatment protocols. The application of OCAs [Fig. 5(c) to 5(e)] increased $Z_{\text{threshold}}$ significantly, while the two control groups, saline + massage [Fig. 5(a)] and PEG-400 + thiazone but no massage [Fig. 5(b)] showed no significant changes. After 15 min of OCAs application assisted with massage, the $Z_{\text{threshold}}$ rose 15% for the OCAs without penetration enhancer and 26% and 31% for PEG-400 with thiazone and 1,2-propanediol, respectively.

4 Discussion

The OCT signal analysis by averaging over all the lateral x positions of three duplicate images gives more information about the depth reflectance, as shown in Fig. 3. The sharp strong reflectance is the specular reflectance from the skin surface and we defined it as $Z = 0$ mm in skin. It is well known that the turbid characteristic of skin results mainly from strong scattering of dermis, so the reflectance from the epidermis is weaker than that from the upper part of dermis for intact skin. And the strong scattering of the dermis makes photons diffuse widely, so it is difficult to detect the signal from inner part of the dermis by OCT. After application of OCAs with a soft massage, agents enter into the dermis and diffuse into the inter-fiber medium or even cause the tissue dehydration for a hygroscopic OCA. This makes the index matching of collagen fibers and medium, and then decreases the scattering of the dermis. More ballistic and quasi-ballistic photons of incident light penetrate deeply into the tissue. Therefore, the deeper OCT image was enhanced and the signal from the upper part of the dermis was weakened after optical clearing of skin in vivo. For both the thiazone and 1,2-propanediol groups combined with massage applied for 15 min, the 300-μm signal increased significantly, 3-fold relative to intact skin, and $Z_{\text{threshold}}$ increased 26% and 31%. The PEG-400 group alone combined with massage caused less clearing, the 300-μm signal increased to 1.5-fold relative to intact skin and $Z_{\text{threshold}}$ increased 15%.

The OCT reflectance depends on two parameters: (1) the attenuation coefficient as photons travel into/out of the skin, and (2) the local reflectivity from each depth position within the skin. This two parameter description has been described by Samatham et al. for confocal reflectance and Levitz et al. for OCT, respectively. The results of this paper show that application of OCAs caused the signal from the superficial dermis to decrease while the signal from the inner dermis increased. Such a decrease in superficial reflectance is likely due to a decrease in local reflectivity. The photon pathlength in/out for signal from superficial dermis is rather short, so the attenuation is not strong. The increase in deeper reflectance is likely due the decrease in attenuation since the photon pathlength in/out is rather long.

Zhong et al. reported similar changes in skin OCT reflectance caused by topical glucose application. Zhong et al. reported that optical clearing on human skin enhanced the OCT signals at all depths. Xu et al. found that optical clearing of porcine skin in vitro increased the reflectance from deeper dermis although the OCT signal from superficial skin did not change. These three previous reports and our current paper are all consistent with OCA inducing an increase in local reflectivity and a drop in attenuation.
The mechanism of optical clearing is still a topic of investigation. Whether the OCA agents cause refractive index changes that shift the refractive index of collagen fibers, or the OCT agents due to their hygroscopic nature cause changes in scattering due to dessication of collagen fibers is still begin explored. Recently, Samatham et al. 38 reported that glycerol caused optical clearing of mouse dermis due to changes in anisotropy rather than the scattering coefficient. The swelling of scatterers in the dermis can lead light scattered in a more forward direction. Alternatively, a hygroscopic OCA induced dehydration may decrease the inter-fiber spacing 34 yielding more tightly packed fibers, which allows more constructive interference between scatterers thereby decreasing the scattering coefficient and increasing the anisotropy of scatter. The relative roles of these possible mechanisms are still under investigation by several investigative groups.

This report illustrates the importance of massage for improving the penetration of PEG-400. Previous investigation showed that the penetration enhancers can significantly improve the permeability of PEG-400 through the stratum corneum. 37 However, when we used a gauze application of PEG-400 with thiazone, the OCT signal rarely changed during the application. Only with sufficient massage during OCAs application can significant optical clearing effect be achieved. Rylander et al. 38 reported that tissue compression induced by mechanical force leads to a reduction of scattering in tissues. Thus, the mechanical force of massage itself can yield an optical clearing effect if compression is kept constant. But the lack of clearing by massage plus application of saline showed that mechanical force by massage was not sufficient for optical clearing. The massage provides mechanical force to reduce the epithelial cell barrier function and allow more OCA to penetrate into the skin. 1,2-propanediol is usually used as a kind of solvent, and its ability as a penetration enhancer has been reported earlier. 28,37,39 This study shows that 1,2-propanediol is as effective as thiazone in enhancing the optical clearing by PEG-400 based on quantitatively analysis.

5 Conclusion
This study demonstrates that the topical application of (1) a mixture of PEG-400, (2) a chemical penetration enhancer, and (3) physical massage can achieve a good optical clearing effect in 15 min on in vivo rat dorsal skin. All three components applied 15 min together can achieve a 3-fold increase in the OCT reflectance from a 300-μm depth and a 31% increase in the image depth Z threshold.

Acknowledgments
The authors are thankful to A. N. Bashkatov, E. A. Genina, N. A. Trunina, and O. V. Glushkovskaya- Semyachkina from SSU for their help in providing experiments. Dan Zhu is thankful for the support of the grants of the National Nature Science Foundation of China (No. 81171376), and the Research Fund for the Doctoral Program of Higher Education of China (No. 20111042110073), Valery V. Tuchin and Dan Zhu appreciate the support of their collaboration by grant NSFC-RFBR for the International Cooperation (No. 3091120074) and RFBR-08-02-92224-NNSF a (RF—China). Valery V. Tuchin is thankful for the support of grants 224014 Photonics4life FP7-ICT-2007-2; RF 2.1.1.4/4989, 2.2.1.1/29501,1.4.09; and RF Governmental contract 02.740.11.0879. RF 1.4.09; RF Governmental contract 02.740.11.0879; and FiDiPro, TEKES (401111/11), Finland.

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