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Abstract. Optical imaging techniques have shown tremendous potential for assessing cutaneous microcirculation, but the imaging depth and contrast is limited by the strong scattering of skin. Current skin windows have to be fulfilled by surgical operation and suffer from some side effects. In this study, a switchable skin window was developed by topical application of an optical clearing agent (OCA) and saline on rat skin *in vivo*. The validity of the skin window was evaluated by the laser speckle contrast imaging technique, and the safety of OCA to the body was tested through histologic examinations. The results indicated that administration of OCA or saline on rat skin *in vivo* can open or close the window of skin repeatedly for three days. With the repair effect of hyaluronic acid and Vaseline, it is able to repeatedly visualize the dermal blood vessels and flow distribution. Long-term observation shows that there is no abnormal reflection in micro-structure, body weight, organ coefficients, histopathologic lesions, or toxic reactions compared with a control group. This switchable window will provide an effective tool not only for cutaneous microcirculation with laser speckle contrast imaging, but also for diagnosis and treatment of peripheral vascular diseases, including tumor research with various optical imaging techniques. [DOI: 10.1117/1.JBO.18.6.061209]

Keywords: skin optical clearing; repeatable imaging; switchable skin window.

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1 Introduction

Some peripheral vascular diseases are tightly coupled into the abnormal structure and function of cutaneous microcirculation.[1] Meanwhile, the abundant microvascular network in the dermis also permits the skin to become a target tissue for establishing tumor models.[2] Thus, the ability to image the structure and function of dermal blood vessels is of considerable importance for investigating changes in cutaneous microcirculation, and tracking tumor angiogenesis, development, and interventional treatment. Optical imaging techniques have a great advantage over other techniques in visualization of microvessels and monitoring of blood flow.[3–8] Among those techniques, the laser speckle contrast imaging (LSCI) technique, a full-field imaging method, can provide both the structural and functional information of blood vessels simultaneously with high spatio-temporal resolution, which has played important roles in mapping cerebral blood flow,[8,9] mesentery microcirculation,[10] etc., but is still limited to transparent tissues.[10–13]

To image dermal microvessels, the dorsal skin chamber (DSC) window is an available model.[2,12–16] With the DSC window, the LSCI has been used in diverse applications, such as evaluation of microvascular response to dermatologic light-based therapies,[13,14] tumor studies,[15] and pharmacokinetics,[16] etc. However, the model is not always satisfactory because the surgical operation is always accompanied by bleeding during the process of establishment and changes in the normal physiological environment of dermal microvessels.[2]

Actually, the tissue optical clearing technique,[17–19] proposed by Tuchin et al., can reduce the scattering of tissue and enhance the light penetration depth by immersion of tissues into optical clearing agents (OCAs). Combination of skin optical clearing with various optical imaging techniques, such as optical coherence tomography (OCT),[20] two-photon microscopy,[21] and second harmonic generation (SHG) imaging,[22] etc., could significantly improve the imaging quality, and acquire more in-depth structural information of skin. Zhu et al. topically treated skin *in vivo* with an OCA and opened a transparent skin window, which allows imaging of both the dermal blood vessels and blood flow distribution with the LSCI technique. After the application of OCA, saline could temporarily close the skin window.[7] However, whether the skin window is switchable for repeatedly imaging the dermal blood vessels has never been reported.

It is well known that the tissue optical clearing is always accompanied by skin dehydration[23] and wrinkling,[24] which may make it difficult to make the skin transparent again. Even though the components of OCA used are biocompatible, the concentrations are much higher than the usual dose in clinical medicine. Previous studies have demonstrated that direct application of OCAs would reduce the blood flow, block the vessels, or even impair the vessels,[11,25–27] and the side effects could be reduced with the decrease of the concentration or dose of OCAs administrated.[11] Zhu et al. found that topical treatment of OCA on the surface of rat skin *in vivo* did not affect the cutaneous blood flow,[7] but whether there are side effects of OCA on

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the dermal blood vessels and blood flow distribution if the OCA was administered to skin repeatedly, needs to be further investigated. Skin is the largest organ of the body and is a significant route for foreign compounds entering the body.[28] After the OCA penetrates the dermis, it will enter the blood and be distributed in various organs of body for the blood circulation. Thus, apart from the influence on blood vessels, both the local toxicity to skin and systemic toxicity to various organs require much attention.

The purpose of this study is to develop a safe, effective, and switchable skin window for repeatedly imaging cutaneous microvessels with an optical clearing method. To avoid the influence of dehydration on skin, some moisturizing and bacteriostatic agents were applied. To assess the safety of OCA to rat skin *in vivo*, the microstructure of skin was observed through histopathologic examinations. Further, to evaluate the potential toxicity of OCA to body, the body weight, organ coefficients, and the histopathologic changes in liver and kidney were also tested.

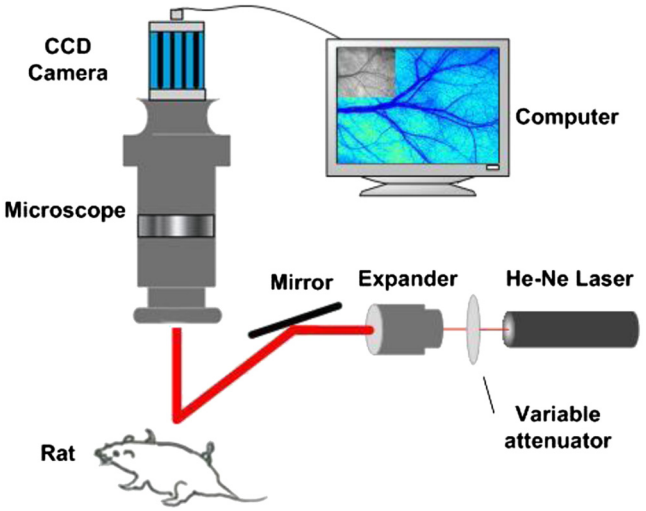

Fig. 1 Schematic of the laser speckle contrast imaging system.

2 Materials and Methods

2.1 *Chemical Agents and Animal Preparation*

The OCA is still based on the previous study,[7] which is composed of two biocompatible agents, PEG-400 and Thiazone, and the volume ratio of PEG-400 and Thiazone is 9 : 1.[29] The refractive index of the mixture solution is 1.47 (WAY-2S ABBE Digital Refractometer, Shanghai YiCe Apparatus & Equipment Co., China). Saline was also used as a control agent.

Animal care and experimental procedures were approved by the Experimental Animal Management Ordinance of Hubei Province, P. R. China. Male Sprague-Dawley (SD) rats ($n = 50$, 105 ± 12 g, 4 to 5 weeks old) were supplied by Wuhan University Center for Animal Experiment (Wuhan, China) and fed under specific pathogen free (SPF) level of feeding condition.

Rats were anesthetized with the mixture of 2% α-chloralose and 10% urethane (8 mL/kg) via intraperitoneal injection. The dorsal hair was shaved and the residual hair was removed with depilatory cream (sensitive hair removal cream, Veet), and then impurities of the region of interest were gently removed by tape stripping 2 to 3 times. After that, rats were randomly divided into two groups: the control group ($n = 10$) and the experimental group ($n = 40$). The experimental group received topical application of warm OCA (37°C), whereas the control group just received warm saline (37°C). The OCA or saline were removed from the skin 30 min later with lukewarm water (37°C). After experiments of the day, rats were back to the standard feeding environment.

In the following 28 days, rats were examined daily for any abnormal appearances of the skin and general reaction, which include awareness, motor activity, posture and muscle tone, etc. The body weight of the rats was recorded before the experiment and once every seven days after treatment of agents.

2.2 *LSCI for Imaging Dermal Blood Vessels*

In this study, the cutaneous microcirculation was monitored by LSCI technique. Figure 1 shows the schematic of LSCI system. A 632.8-nm He-Ne laser (3 mW) was used to illuminate the interested area with a beam expanded through a collimating lens. A sequence of raw speckle images was then imaged through a stereo microscope (SZ61TR, Olympus, Japan) onto a charge coupled device (CCD) camera (Pixelfly USB, PCO Computer, Germany). The CCD exposure time used was 5 ms. Because the laser speckle temporal contrast analysis (LSTCA) method was reported to be highly effective for suppressing the influence of static speckle from the scattering medium,[9] it was used here to obtain the two-dimensional (2-D) blood flow distribution maps of cutaneous microvessels.

Forty rats were used for dermal blood flow imaging. After the prepared animal model was placed on the experimental platform, the initial raw speckle images of the interested skin area were obtained. Then, the OCA was topically applied on rat dorsal skin *in vivo*, and a sequence of raw speckle images were obtained at 2-min intervals. After 30 min, the OCA was gently removed and the saline solution was topically applied on the same area for temporary recovery. The raw speckle images were taken again. During the intervals, a digital camera (T200, Sony, Japan) was used to take the white-light photos. To reduce the side effects of OCA on rat skin, after the interested skin area was washed by saline solution, 0.5% hyaluronic acid (HA) and Vaseline were topically applied to keep skin moist and to prevent the contact of skin and bacterium.[30–32] On the second and third day, the above experimental procedure was performed repeatedly.

2.3 *Histopathologic Examinations for Evaluating the Safety of OCA*

Ten animals on the first day, the 14th day, and the 28th day after treatment of OCA for three days, were sacrificed, respectively. The skin of interest was cut and immersion-fixed in 4% neutral formaldehyde, and then dehydrated and embedded in paraffin. After cutting into 4-μm sections, the samples were then stained with hematoxylin and eosin (H&E), and Masson, respectively, and examined under a light microscope (IX71, Olympus, Japan).

On the 28th day after treatment of OCA and saline for three days, 10 rats from the experimental group and 10 from the control group were sacrificed, and various organs, such as liver, heart, spleen, kidney, brain, and lungs were removed and weighed. The organ coefficient was obtained by dividing the weight of the organ by body weight.

Both liver and kidney are important target organs of toxic effects of chemicals because they are primarily involved in metabolism and excretion of chemicals.[33] In this work, the liver and kidney tissue were also tested by histopathologic examination with H&E staining.

3 Results

3.1 *OCA Induced Switchable Skin Window on Rat In Vivo*

The OCA was repeatedly applied on the same area of rat skin *in vivo* in the following 3 days, and the typical results are shown in Fig. 2. Figure 2(a) shows the morphologic photos of the skin before, 30 min after application of OCA, and recovery with saline. Figure 2(b) shows the corresponding laser speckle temporal contrast images, which demonstrate the flow distribution information of the dermal blood vessels.

It can be seen that on the first day topical application of OCA on rat skin *in vivo* makes the skin become transparent, which allows to image the dermal blood flow by the LSCI technique; whereas treatment with saline makes the skin recover to its initial state, which is consistent with the previous study.[7] On the second or the third day, the initial state of rat skin is almost the same as the intact skin, without wrinkles and edema occurring in the skin. It is difficult to image blood flow because the skin is turbid. After topical application of OCA on the same place, the skin becomes transparent again, and even the small branches of dermal blood vessels can be clearly observed. The details of dermal blood-flow distribution information are available to be acquired by LSCI technique with high resolution. With treatment of saline solution, the skin recovers to the initial state, and the blood vessel structure and flow information are concealed by the high scattering of skin once again. Comparing the transparent skin in the following two days with that of the first day, we can find that the vessel structure and flow distribution of dermal blood vessels are quite consistent.

To further quantitatively evaluate whether repeated administration of OCA affects the dermal blood vessels, the diameter of some typical vessels (pointed out by arrows 1–4) in Fig. 2 are calculated, and the results are summarized as mean ± standard deviation in Table 1. In addition, a one-factor analysis of variance (ANOVA) was applied for statistical analyses, and the differences in vessel diameter between the following two days and the first day were inspected by least significant difference (LSD) test, respectively. It can be found that, after repeated application of OCA on rat skin, the diameters of dermal blood vessels measured in the following two days are essentially unchanged from that measured in the first day, and there are no significant changes in diameter between day 2, day 3, and day 1

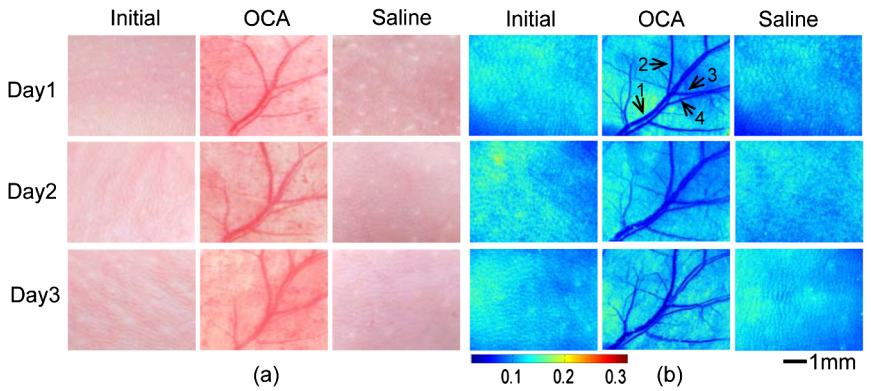

Fig. 2 Typical visual photos (a) and corresponding laser speckle temporal contrast images (b) of *in vivo* rat skin. The arrows 1 to 4 indicate the interested blood vessels.

Table 1 Measurement of diameter of dermal blood vessels shown in Fig. 2.

Dermal blood vessels		Day 1	Day 2	Day 3
Vessel 1	Diameter (μm)	175.69 ± 4.60	176.10 ± 4.33	175.28 ± 9.13
	P value	—	0.91	0.91
Vessel 2	Diameter (μm)	128.18 ± 3.96	129.41 ± 3.86	129.82 ± 3.96
	P value	—	0.57	0.45
Vessel 3	Diameter (μm)	127.36 ± 2.26	129.41 ± 1.98	127.78 ± 2.80
	P value	—	0.12	0.57
Vessel 4	Diameter (μm)	95.83 ± 2.80	95.83 ± 3.25	92.69 ± 3.47
	P value	—	1.00	0.09

($P > 0.05$). That is, the OCA has no obvious side effects on the dermal blood vessels. Thus, it seems to be a switchable window that topical administration of OCA or saline on rat skin *in vivo* can open or close the skin window. It should benefit from the excellent moisturizing and bacteriostatic efficacy of HA and Vaseline, which permits the blood vessel structure and flow distribution to be imaged repeatedly without surgical operation.

3.2 *Short-Term and Long-Term Effects of OCA on the Microstructure of Skin*

The above results demonstrate that a switchable skin window can be established with the optical clearing method, but the safety of OCA to the skin and body need to be evaluated. Figure 3 shows the results of histopathologic examinations of rat skin before and after treatment by OCA. The H&E stains of skin sections in the top row show the microstructure of skin, and the Masson stains of skin sections in the bottom row show the collagen alignment in dermis. Figure 3(a) and 3(e) shows the control skin sections; others in Fig. 3 show the skin sections on the first day, the 14th day, and 28th day after treatment of OCA for three times, and the arrows 1 to 3 in Fig. 3(a) point out the structure of the stratum corneum, epidermis, and dermis, respectively.

It can be found that, for control skin, the structure of epidermis and dermis is integrated. On the third day, after treatment of OCA for 30 min, the stratum corneum is destroyed, and no inflammation cells are found in epidermis and dermis. Moreover, the epidermis becomes thin, whose thickness decreases from $59.36 \pm 8.68\,\mu$m to $21.06 \pm 2.31\,\mu$m. However, on the 14th day after treatment by OCA for three days, the stratum corneum of rat skin recovers to normal. On the 28th day after treatment, the good condition remains, and there are no obvious differences in the microstructure of rat skin between the control skin and the OCA-treated skin.

Previous studies showed that the process of skin optical clearing might be accompanied by structural modification or dissociation of collagen.[34] Hence, Masson stains of skin sections after application of OCA were also conducted to see the changes in collagen alignment. The results show that, for control skin, the collagen is arranged densely. After application of OCA for three days, the arrangement of collagen is unchanged compared with the control skin. On the 14th day and the 28th day after treatment with OCA three times, the collagen alignment is also unchanged. That is, no histopathologic or microstructural changes in rat skin are induced by administration of OCA.

3.3 *Effects of OCA on the Body and Organs*

Further, we performed toxicity experiments to demonstrate whether there is potential toxicity of OCA to the rat body and various organs.

To evaluate the influence of OCA on the body and target organs, the weights of body and various organs, i.e., liver, heart, spleen, kidney, brain, and lungs, etc., were obtained, and the organ coefficients were calculated. Data of body weight and organ coefficients were summarized as mean ± standard deviation in Table 2. A one-factor analysis of variance (ANOVA) was applied for statistical analyses, and the differences between the experimental group and control group were inspected by the Q method (student-Newman-Keul test). It can be found that the body weight has almost doubled in the four-week observation period, but there are no significant differences in body weight and organ coefficients between the control group and the experimental group ($P > 0.05$).

Furthermore, on the 28th day after treatment of OCA or saline, both the kidney and liver were histopathologically

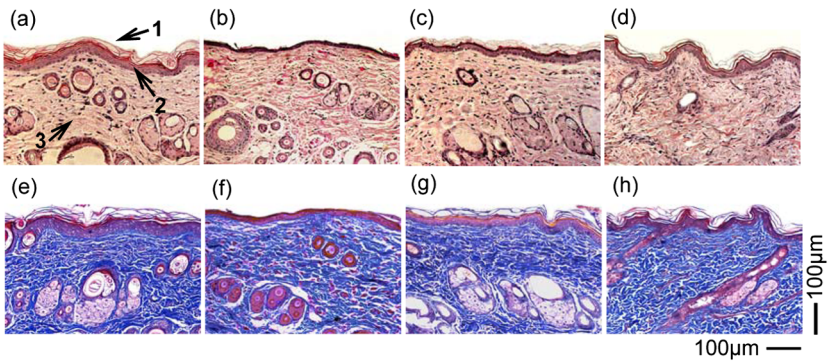

Fig. 3 H&E stains (top row) and Masson stains (bottom row) of tissue sections of rat skin. (a) and (e) Control skin sections; (b) and (f), (c) and (g), and (d) and (h) skin sections on the first day, the 14th day, and the 28th day after treatment of OCA for three times. The arrows 1 to 3 point out the stratum corneum, epidermis, and dermis, respectively.

Table 2 Measurement of body weight and organ coefficients of rat after treatment with OCA.

Groups	Initial body weight (g)	Final body weight (g)	Heart (%)	Liver (%)	Spleen (%)	Lung (%)	Kidney (%)	Brain (%)
Control	112 ± 9.08	211 ± 12.45	0.35 ± 0.01	3.60 ± 0.40	0.23 ± 0.04	0.55 ± 0.02	1.03 ± 0.11	0.93 ± 0.05
OCA	103 ± 6.71	200 ± 12.25	0.34 ± 0.02	3.63 ± 0.52	0.22 ± 0.04	0.54 ± 0.04	0.92 ± 0.05	0.92 ± 0.04

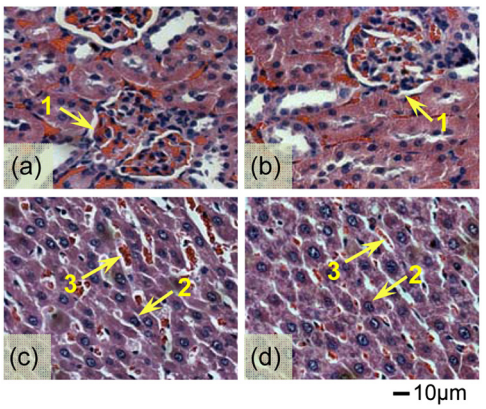

Fig. 4 H&E stains of kidney (top row) and liver tissue (bottom row). (a) and (c) Control tissue sections; (b) and (d) Tissue sections on the 28th day after treatment with OCA for three days. The arrows 1 to 3 point out the structure of glomeruli, hepatocytes and sinusoid, respectively.

examined by H&E staining, respectively. Figure 4 shows the typical sections of kidney (top row) and liver (bottom row). The left column shows the control samples and the right column shows the experimental samples. It can be seen that after treatment by OCA, the cortical architecture of kidney tissue remains intact. Compared with the control section, neither inflammatory infiltrate nor abnormal histopathologic changes were observed in glomeruli pointed out by arrow 1 in Fig. 4(a) and 4(b). Compared with normal liver tissue, the OCA-treated hepatic tissue section has no pathological abnormalities. The hepatocytes pointed out by arrow 2 are arranged in organized plates with well-formed sinusoids pointed out by arrow 3 in Fig. 4(c) and 4(d) in between.

4 Discussion and Conclusions

The above results indicate that a switchable skin window can be established by topical application of OCA on rat skin *in vivo*, which enables the dermal blood vessels and flow distribution to be imaged repeatedly by the LSCI technique without surgical operation. The results also demonstrate that treatment of OCA does not affect the structure and flow distribution of dermal blood vessels. Successful establishment of the switchable skin window should be due to the repair effect of HA and Vaseline. HA is naturally biocompatible, biodegradable, and lacks immunogenicity. It is a component of extracellular matrix and has high capacity of water-sorption and water retention, which has been widely applied to promote wound healing and preserve moisture, etc.[30,31] Vaseline is a mineral wax, which has been conventionally used for wound healing. It can put the skin in its best condition, and hence improve the skin's ability to repair. Meanwhile, it can also block contact between skin and bacterium.[31,32] The two agents act concurrently on rat skin, which permit the skin to recover quickly from dehydration.

In the previous investigations, glycerol and glucose were commonly used OCAs of skin,[26,27] but it is difficult for the two agents to penetrate the epidermis into the dermis, so the optical clearing of skin used to be realized by dermal injection of OCAs.[27] However, direct action of OCAs on blood vessels of cortex,[25] dermis,[26] mesentery,[27] and chick chorioallantoic membrane[11] would induce some side effects, such as decreased blood flow velocity, blockage or impairment of blood vessels, and side effects depended on the concentration or dose of OCAs.[11] In this work, the use of a chemical penetration enhancer, Thiazone, could enhance the penetration of OCA in the dermis by topical application of OCA on the surface of skin, and produce optimal optical clearing efficacy. However, the concentration and dose of OCA that reaches to dermal blood vessels is much lower than those through direct application. Therefore, there is no obvious side effect on dermal blood vessels and blood flow even with repeated treatment.

Of course, the application of OCA destroys the stratum corneum and makes the epidermis become thin temporarily, which may be attributed to the penetration enhancing effect of thiazone and the dehydration of skin, but no long-term pathologic changes in microstructure were found in the skin tissue. It is well known that body weight and organ coefficients are both important indices of toxicity, which reflect the toxic effects of drugs and other substances on body and target organs.[35] An increase in organ coefficient means that some hyperemia, edema, hyperplasia, hypertrophy, etc., may occur in the target organ. Conversely, a decrease in organ coefficient indicates viscera atrophy, ansplasia, etc. The above results demonstrate that treatment of OCA does not induce abnormalities in the rat body and various organs. In addition, both the liver and kidney are the major organs for metabolizing drugs, meanwhile, the former is responsible for toxic reaction, and the latter for excreting toxic substances. Meanwhile, these two organs are usually vulnerable to drugs or some toxic substances.[33] From their organ coefficients to histopathologic examinations, it can be concluded that no inflammatory or toxic reactions were induced by administration of OCA. Thus, the switchable skin window has long-term safety.

Here, the switchable skin window provides an innovative model for imaging cutaneous microcirculation repeatedly. Compared with the DSC model,[2] the optical clearing method induced switchable skin window does not need surgical operation. Thus, the growth of granulation tissue and inflammatory reactions induced by an open wound can be avoided. In addition, in the DSC model, the cover glass creates an artificial surface, which may alter the cell activities, such as motility.[2] In contrast, the switchable skin window can be opened and closed just by administrating OCA or saline solution, so the artificial surface is avoided. When the DSC model is applied to tumor study, even though the subcutaneous tumor can be observed directly and repeatedly, the observation window of about 11 mm usually limits the tumor size.[2] For the switchable skin window, this limitation can be avoided because the size and location of the observed skin area is defined optionally.

It cannot be denied that the switchable skin still faces with challenges for repeatable imaging based on the optical clearing method. For instance, the thickness of adult rat skin will increase with age and weight, and the optical clearing efficacy of skin will be limited because the penetration of OCA into dermis will become more difficult. In addition, repeated administration of OCA can result in the stripping of the stratum corneum and dehydration, which will make epidermis become thin quickly. In this case, epidermal cell proliferation will occur, and then make the epidermis thicker in the next days. This will cause difficulty for repeatedly imaging dermal blood vessels and flow even though there is no long-term toxicity to the body. Therefore, further research needs to be performed on the development of the optical clearing method and better medicine for repairing the skin.

In any case, a switchable skin window is developed based on topical application of OCA on rat skin *in vivo* for three days, which provides a method for repeatedly imaging the dermal blood vessels and flow distribution information by using LSCI technique with high resolution, and safety assessments show that the optical clearing solution is safe to the skin and body. Further, if this window could be combined with other optical imaging techniques, i.e., OCT[20,36] and fluorescence imaging,[37] more exciting achievements could be obtained, which would be quite beneficial for diagnosis and treatment of peripheral vascular diseases and tumor research.

It should be noted that the tissue optical clearing technique is a universal method for improving optical imaging depth. Therefore, it is necessary to accurately evaluate the depth of optical clearing effect achieved. For skin, the strong scattering is mainly from the dermis. After topical treatment of OCA on skin, the agents will pass through epidermis to dermis, replace the water of dermis, and then make the skin transparent. With optical clearing of skin, the dermal blood vessels and blood flow could be monitored easily with the LSCI technique. However, whether the OCA penetrates into deeper tissue, further investigations need to be carried out.

Acknowledgments

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