Monitoring photo-thermal response and collagen remodeling of mouse skin with multiphoton microscopy

Shulian Wu (吴淑莲), Hui Li (李 晖)*, and Xiaoman Zhang (章小曼)

Key Laboratory of OptoElectronic Science and Technology for Medicine, Ministry of Education, Fujian Provincial Key Laboratory of Photonic Technology, School of Physics and OptoElectronics Technology, Fujian Normal University, Fuzhou 350007, China

**E-mail: hli@fjnu.edu.cn*

Received January 11, 2010

Two-photon excitation fluorescence (TPEF) and second-harmonic generation (SHG) are detected through multiphoton microscopy (MPM). The major signals have the potential to monitor the process of tissue changes. TPEF and SHG are used to monitor the skin photo-thermal response to irradiation with intense pulsed light sources (λ is in the range of 560−1200 nm) and trace the process of skin remodeling *in vivo* at different time intervals. TPEF intensity is nearly unchanged at different time intervals after irradiation, whereas SHG intensity changes considerably. The results reveal the photo-thermal effect of nonablative light sources and the process of collagen remodeling at the sub-micron level.

OCIS codes: 170.1870, 170.3880, 180.4315.
doi: 10.3788/COL20100808.0784.

Multiphoton microscopy (MPM)[1] is an optical imaging technology that can perfectly combine two-photon excitation fluorescence (TPEF) and second-harmonic generation (SHG) with high spatial resolution. Since its introduction by Denk *et al.*[2], TPEF originating from intrinsic sources (e.g., cofactors, proteins, elastic fibers, nicotinamide adenine dinucleotide, and so on) has been widely used for cell and tissue imaging[3]. SHG is a coherent nonlinear optical process involving no electronic excitation, and has also been successfully used to image non-centrosymmetric structural proteins, such as collagen, myosin, and thyroid tissue[4−6].

In the past decades, with the introduction of selective thermolysis by Anderson *et al.*[7], nonablative skin rejuvenation has become increasingly popular among the aging population who desire for a fresher and younger appearance. With laser or intense pulsed light (IPL), skin collagen selectively absorbs incident light that generates heat, thereby causing fiber shrinkage[8] and further resulting in tighter and smoother skin[9]. However, the processes of denaturing, remodeling, and tightening collagen are still unclear, and many complications have occurred in clinics[10].

Recently, MPM has been proposed for the analysis of mouse skin irradiated by ablative laser[11], which presented the skin immediate response to different doses of Er:YAG laser. Conversely, we devote to studying nonablative light sources, such as IPL, whose irradiation effect on skin differs from that of ablative laser. We monitor the process of nonablative collagen remodeling of injured skin using TPEF and SHG. The present study aims to provide further understanding of the mechanism of collagen remodeling after nonablative photo-thermal injury.

Eight mature Kunming mice (12 weeks old) were chosen, and all procedures involving mice tissues were approved by the Institutional Animal Care and Use Committee. Each mouse was anesthetized by intraperitoneal phenobarbital injection after dorsal hair was removed[12]. The dorsal skin was then stabilized in a fold chamber, contracted with a cover slip, and then observed by MPM before irradiation. The dorsal skin was again observed at different durations (2 minutes, 1 hour, 6 hours, 12 hours, 1 day, 1.5 days, 3 days, 5 days, 9 days, and 22 days) after irradiation by IPL Queen Premium, λ is in the range of 560−1200 nm). Subsequently, a spot of about 8×34 (mm) in the dorsal skin was irradiated using the fluence of 34 J/cm^2, pulse durations of 2.8 and 3.4 ms, and pulse delay time of 40 ms. The parameters adopted were based on our previous study[13]. The dorsal skin of each mouse, a region of about 5×20 (mm) within the IPL spot size, was observed. Stack images from each region were obtained at different time intervals. Each time, the unirradiated region symmetrical to the irradiated region was used as calibration reference, and the ratio of average intensity in the unirradiated region was kept constant by adjusting the detected gain. For the purposes of quantitative analysis, we randomly selected 10 rectangular areas (200×200 (μm)) in the irradiated region for each interval and each mouse. Test statistics was performed using SPSS 10.0.

The MPM system used is based on the combination of an Axiovert 200 inverted microscope with Zeiss LSM 510 META laser scanning microscopy and a Coherent Mira 900-F mode-locked femtosecond Ti:sapphire laser (110 fs, 76 MHz), tunable from 700 to 980 nm[12]. The excitation wavelength of 850 nm was used with the average power of about 10 mW irradiated on the sample spot. An oil immersion objective (63×, numerical aperture (NA) = 1.4) was employed, and the sizes of all images were of 512×512 (pixels). TPEF and SHG were generated and collected from the sample focal plane. The SHG signal was detected at 425 nm at the center with the bandwidth of 20 nm, whereas the TPEF was detected by a band-pass filter for fluorescence detection between 450 and 714 nm.

The high-resolution MPM images obtained by the

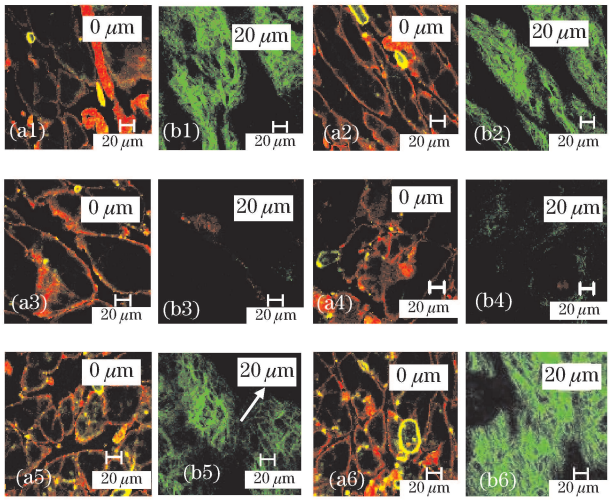

Fig. 1. TPEF and SHG images of collagen. (colorful online). The red channel represents TPEF, and the green channel represents SHG. (a) 0 μm on the skin surface; (b) 20 μm below the skin surface, representing the collagen fibers. Briefly, (a1) and (b1) before irradiation; (a2) and (b2) 2 minutes after irradiation ; (a3) and (b3) 1 day after irradiation; (a4) and (b4) 3 days after irradiation; (a5) and (b5) 9 days after irradiation; (a6) and (b6) 22 days after irradiation.

TPEF/SHG signals from the mouse skin at 11 different intervals are partly shown in Fig. 1(a). The figures clearly show both the stratum corneum represented by autofluorescence and the morphology of corneocytes. TPEF and SHG intensities, which originate from the skin surface, are very strong and almost unchanged with time, whether the skin is irradiated or not. This could be the protective effect of inner-couplers cooling in the IPL apparatus. Meanwhile, SHG intensity changes considerably at different intervals; this can be explained by the theory of selective photothermolysis[7]. When a light beam reaches the dermis through the epidermis, the ensuing heat results in collagen coagulation and then later, necrosis.

Before irradiation, the collagen is loose. The strong SHG signals emitted by collagen fibers in the dermis can be clearly observed in Fig. 1(b1). Two minutes after irradiation, SHG intensity decreases slightly, but it is still evident and easily observable (Fig. 1(b2)). This means that thermal absorption does not denaturize the dermis completely and immediately. From 1 hour to 1 day, SHG intensity decreases considerably. The inflexion point appears on one day when SHG is at its lowest intensity (Fig. 1(b3)). The fibrous morphology of collagen is disrupted by thermal denaturization, and its structure is almost diminished. At the same time, a bit of collagen is replaced by some fluorescent materials (as shown by the arrows). The SHG signal is still detectable from the dermal collagen, although its intensity decreases to a large extent. This decrease is caused by the fluorescent shielding effect from the disrupted corneocytes. After 3 days, weak SHG signals of collagen are found, indicating that the collagen is remodeling gradually (Fig. 1(b4)). Compared with SHG intensity 1 day after irradiation, SHG intensity after 3 days increases but is still weak. This seems to indicate an increase in the quantity of remaining collagen in the injured region, that is, the injured collagen regenerates. The quantity of collagen increases and the collagen structure becomes distinct 9 days after irradiation (Fig. 1(b5)). After 22 days, SHG intensity is higher than that prior to irradiation, collagen quantity is higher, and collagen tightness is much firmer than those in unirradiated regions (Fig. 1(b6)). Collagen tightness is based on the spaces between collagen bundles in the Zeiss software "profile". After remodeling is completed, the intensity of the SHG signal is stronger than that in the unirradiated region.

It has been reported that a change in SHG intensity is a good predictor of collagen structural disruption[14]. We obtained the SHG spectra of skin collagen from 11 different intervals by lambda scanning mode. The average data in each interval were normalized to the maximal peak intensity (Fig. 2(a)). Normalized intensity versus renovated time is shown in Fig. 2(b).

Before IPL irradiation, the value of SHG intensity reaches about 0.8, and decreases gradually to the lowest (i.e., less than 0.1) 1 day after irradiation (Fig. 2). Then, the intensity increases daily until the highest (i.e., about 1) after 22 days, which is greater than that in the unirradiated group. SHG intensity in collagen denotes the quantity of collagen during the processes of irradiation and renovation. The results are statistically significant ($P < 0.05$).

To further study the changes in intensity, we quantitatively analyzed SHG intensity with depth at different intervals. Figure 3 shows seven typical time points of SHG intensity in a rectangular area in each stack image, which was obtained by Zeiss software.

The strongest SHG intensity before irradiation is at about 20 μm below the skin surface. After 1 day, the lowest depth is about 14 μm. After 22 days, the depth is 24 μm. These changes in depth may be due to the variability

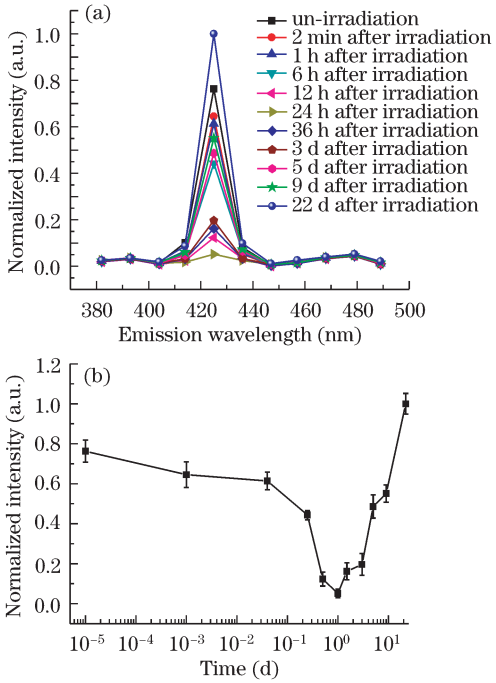

Fig. 2. Intensity of collagen spectra (depth = 20 μm). (a) Normalized intensity of SHG versus emission wavelength; (b) normalized intensity of SHG intensity versus logarithm of time.

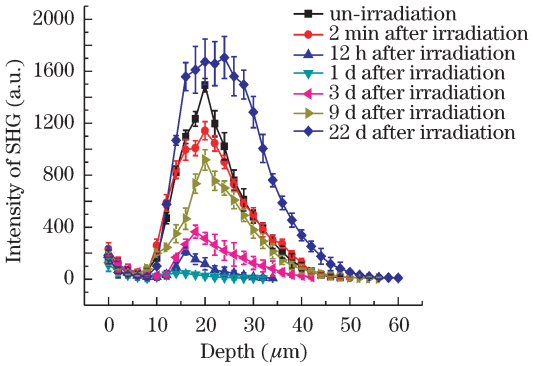

Fig. 3. Intensity of SHG with the depth of skin. Error bars represent standard deviation (SD) in the average values of eight skin samples.

of effective optical attenuation coefficient during the processes of injury and renovation, and skin pigmentation considerably increases the absorption. The total detectable depth in mouse skin also changes during the process. Before irradiation, the detectable depth is about 50 μm. One day after irradiation, the detectable depth decreases to the lowest value of about 36 μm. The decrease in detectable skin thickness, is due to the contraction mediated by fibroblast cells[15]. Twenty-two days after irradiation, the depth increases to about 60 μm. From Fig. 3, we can observe that the detectable depth increases with SHG intensity.

In conclusion, MPM with SHG and TPEF is used to monitor the process of photo-thermal damage by IPL, and remodeling in mouse skin *in vivo*. Experimental results show that the approximate values of TPEF and SHG signal intensities prior to irradiation are maintained immediately after IPL irradiation; the values change little during the process of remodeling in the skin surface. On the other hand, the SHG signal at 20 μm below the skin surface changes considerably. One day after irradiation, the signal reaches the lowest value, and then the signal intensity increases gradually. Twenty-two days after irradiation, the intensity of the SHG signal is much higher than that in the unirradiated region. The detected depth is also deeper than that previously observed. In short, MPM is a promising method for the noninvasive monitoring of optical thermal healing *in vivo*.

This work was supported by the Research Fund for the Doctoral Program of Higher Education (No. 200803940001), the Natural Science Foundation of Fujian Province (No. 2010J01323), and the National Natural Science Foundation of China (No. 60578056). The authors especially thank Dr. Y. Li for discussions.

References

1. R. Gauderon, P. B. Lukins, and C. J. R. Sheppard, Micron. **32,** 685 (2001).
2. W. Denk, J. H. Strickler, and W. W. Webb, Science **248,** 73 (1990).
3. X. Qu, J. Wang, C. Yao, and Z. Zhang, Chin. Opt. Lett. **6,** 879 (2008).
4. R. M. Williams, W. R. Zipfel, and W. W. Webb, Biophys. J. **88,** 1377 (2005).
5. J. Chen, S. Zhuo, T. Luo, and J. Zhao, Chin. Opt. Lett. **4,** 598 (2006).
6. Z. Li, Z. Huang, R. Chen, C. Li, S. Lin, and Y. Chen, Chinese J. Lasers (in Chinese) **36,** 765 (2009).
7. R. R. Anderson and J. A. Parrish, Science **220,** 524 (1983).
8. T. R. Lyons, P. L. Griffth, F. H. Savoie, and L. D. Field, Arthroscopy. **17,** 25 (2001).
9. R. E. Fitzpatrick, M. P. Goldman, N. M. Satur, and W. D. Tope, Arch. Dermatol. **132,** 395 (1996).
10. A. Willey, R. R. Anderson, J. L. Azpiazu, A. D. Bakus, R. J. Barlow, J. S. Dover, J. M. Garden, S. L. Kilmer, N. Landa, D. Manstein, E. J. Ross, N. Sadick, E. Tanghetti, D. Yaghmai, and B. D. Zelickson, Lasers Surg. Med. **38,** 1 (2006).
11. T.-H. Tsai, S.-H. Jee, J.-Y. Chan, J.-N. Lee, W.-R. Lee, and C.-Y. Dong, J. Biomed. Opt. **14,** 024034 (2009).
12. S. Wu, H. Li, Z. Xiao, and S. Xie, Proc. SPIE **7280,** 728022 (2008).
13. S. Wu, H. Li, and S. Xie, Acta Laser Biology Sin. (in Chinese) **18,** 320 (2009).
14. S. Yen, W.-L. Chen, S.-J. Lin, S.-H. Jee, Y.-F. Chen, L.-C. Lin, P. T. C. So, and C.-Y. Dong, Biophys. J. **91,** 2620 (2006).
15. A. T. Yeh, B. Kao, W. G. Jung, Z. Chen, J. S. Nelson, and B. J. Tromberg, J. Biomed. Opt. **9,** 248 (2004).