Quantitative analysis on collagen morphology in aging skin based on multiphoton microscopy

Shulian Wu
Hui Li
Hongqin Yang
Xiaoman Zhang
Zhifang Li
Shufei Xu

Quantitative analysis on collagen morphology in aging skin based on multiphoton microscopy

Shulian Wu, Hui Li, Hongqin Yang, Xiaoman Zhang, Zhifang Li, and Shufei Xu

Fujian Normal University, School of Physics and OptoElectronics Technology, Institute of Laser and Optoelectronics Technology, Fujian Provincial Key Laboratory of Photonics Technology, Key Lab of OptoElectronic Science and Technology for Medicine, Ministry of Education, Fuzhou 350007, China

Abstract. Multiphoton microscopy was employed for monitoring the structure changes of mouse dermis collagen in the intrinsic- or the extrinsic-age-related processes *in vivo*. The characteristics of textures in different aging skins were uncovered by fast Fourier transform in which the orientation index and bundle packing of collagen were quantitatively analyzed. Some significant differences in collagen-related changes are found in different aging skins, which can be good indicators for the statuses of aging skins. The results are valuable to the study of aging skin and also of interest to biomedical photonics. *© 2011 Society of Photo-Optical Instrumentation Engineers (SPIE)*. [DOI: 10.1117/1.3565439]

1 Introduction

The skin is a complex multilayered structure. It not only performs numerous physiological functions vital to body homeostasis, but also acts as the first defense zone of body against extrinsic factors. With the development of dermatology, the process of cutaneous aging and its underlying mechanism have attracted worldwide attention.[1–5] Cutaneous aging is a complicated biological process affecting different constituents of skin, which can be divided into two types: chronological aging and photoaging.[2,3]

Recently, various optical methods, such as multiphoton microscopy (MPM)[6,7] serving as two photon excited fluorescence and second harmonic generation (SHG) microscopy, optical coherence tomography,[8,9] confocal laser scanning,[9,10] and diffuse spectroscopy[11] have been developed for use in some medical applications and in dermatology.[8–16] Since MPM is a label-free noninvasive approach with high-resolution, it has been successfully used as an effective technique in dermatology, including aging skin.[14–16] However, the effects of the above two cutaneous aging processes are often overlapped including changes in epithelium and dermis. The mechanism of the cutaneous aging

process is still elusive. To the best of our knowledge, no one has investigated the collagen alignment of different statuses of aging skin *in vivo*, particularly in different depths and sections of the collagen morphology, which is very important to reveal the characteristics of the aging process.

In this study, we used MPM (Ref. 12) in combination with fast Fourier transform (FFT) to determine the morphological parameters of the chronological aging with young and old stages versus photoaging skins of mice *in vivo*.

2 Materials and Methods

Four groups (ten in each group) of Kun-ming mice with ages of 8, 16, 50, and 60 weeks, respectively, were chosen as chronological aging models. Then ten mice, aged six weeks, were irradiated by ultraviolet light (the doses of UVA and UVB are 78 and 9.72 J/cm^2, respectively[11]) for ten weeks, which is regarded as photoaging models. The experimental system was briefly described.[12] The excitation wavelength at 850 nm was used with an average power of about 8 mW. An objective with Plan-Neofluar ($\times 10$, NA $= 0.3$) was employed. The signal was detected at 425 nm with a bandwidth of 20 nm. All images are of 512×512 pixels. Since the surface of mouse skin *in vivo* is not absolutely flat, a zero depth (0 μm) was defined as the position where the multiphoton signals reflecting from the interface between the skin tissue and the glass cover-slip reached maximum. This zero depth was considered as the skin surface in our study. The SHG images of dermal structure from the skin surface to the depth of 80 μm were detected *in vivo*.

A system (online at http://rsb.info.nih.gov/ij) offers FFT analysis for imaging processing that has been used in much research.[15–18] In this study, the ratio of short axe to long axe of the generated power plot of images was used to estimate the collagen orientation index (N), which was calculated by $N = [1-(\text{short/long})]$.[16,17] The N of perfectly random tissue is "0," meaning isotropic behavior, and its FFT plot is a circular. The FFT analysis with parallel collagen orientation yields an elongated power that leads to a long orientation index (maximum is "1").[17,18] Furthermore, we introduce a parameter L, denoting the collagen bundle packing, expressed as $L = 512*(1/h)$,[17] where h is the pixel distances between the centers of gravity of two first-order maxima in 3D images. The value of L represents the periodicity in collagen. Twenty images were randomly selected for the FFT analysis to obtain the values of N and L in 8, 16, 50, 60 weeks, and photon-induced aging, respectively. The experimental results were compiled from a statistical test with SPSS 15.0 software (SPSS Inc., USA).

3 Results and Discussion

Figures 1(a)–1(c) are the SHG images that give the visualization of collagen structure status at different depths where the SHG signals are most strong among the different aging processes. From Fig. 1, we can see the depth is about 56 μm below skin surface in the young stage (8 to 16 weeks) and in the old stage (50 to 60 weeks) the depth decreases to 48 μm. While for photoaging, this depth is about 40 μm. Therefore, the collagen statuses are age-related. It will decrease with age or suffering

Address all correspondence to: Hui Li, School of Physics and OptoElectronics Technology, Fujian Normal University, Fuzhou, Fujian 350007 China. Tel: 8659122868133; Fax: 8659122868133; E-mail: hli@fjnu.edu.cn.
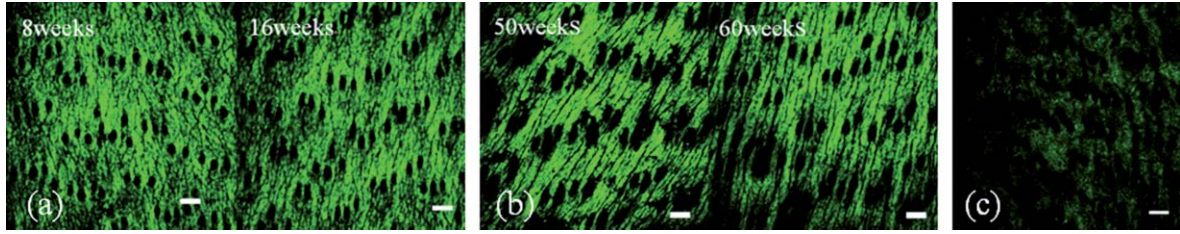
Fig. 1 Visualization of collagen structure status in the skin of different aging models, scale bar $= 100 \ \mu$m. (a) Young stage of chronological aging, including 8 to 16 weeks; (b) old stage of chronological aging, including 50 to 60 weeks; (c) the photoaging.

from UV light irradiation. The collagen displays a denser matrix and the bundles are much tighter at the young stage. It shows that the collagen is a good indicator for visualizing the precise status of dense matrix and the bundles inside the collagen. This finding is consistent with other works.[13,14]

The intensities and periodicity within the images can be extracted through FFT analysis. Figures 2(a)–2(c) give the power plots of FFT in different aging stages. It can be seen that the shorter ellipse and larger area of the plot is at the young stage, which indicates that collagen bundles arrange more randomly and the intensity is stronger for younger skin. The plot is a longer ellipse for intrinsic-age-related skin, indicating a higher orientation of collagen bundles. The ellipse is the shortest and the area of the plot is the smallest for extrinsic-age-related skin, implying a random array of collagen bundles that was a great loss. The results are also consistent with other studies.[15–17]

Then, the value of N in different stages of dermis skin were calculated and summarized in Table 1. Concretely, they are about 0.4 and 0.75 in young and chronological aging stages, respectively. This shows N increases with age. But for photoaging skin, the value of N is about 0.3, which is less than that of the young stage. The abnormal phenomenon probably resulted from the damage of collagen structure in photoaging skin. It reveals that

the mechanism varies with different types of aging in collagen alignment.

To intuitional visualization of intensities and periodicity within the SHG images, 3D images of the FFT plot images of different aging skins, are given in Fig. 3. The values of h are obviously displayed in Fig. 3. We can find that it is larger for younger skin than other statuses of skin aging. While for photoaging skin, h is the smallest. Similarly, the calculations of the value of L in different statuses of aging are given in Table 1. They are about 12 and 18 for young and chronological aging skin, respectively. It indicates L increases with age in the chronological aging process. While the value for photoaging skin is one time larger than in the young stage skin. Since L denotes the collagen fibrils periodicity, the results indicate that the collagen bundle is tighter in the young stage and the photoaging skin is the most incompact.

Figure 4 gives the changes of N versus detectable depths. We can see it obviously changes in the young stage. It is larger in the upper dermis collagen layer and it gradually decreases with depth until it reaches the strongest SHG intensity. The result is consistent with other study on histopathology of scar tissue.[17] For chronological aging skin, the N slightly changes with a high value. As for the photoaging skin, it is nearly unchanged with
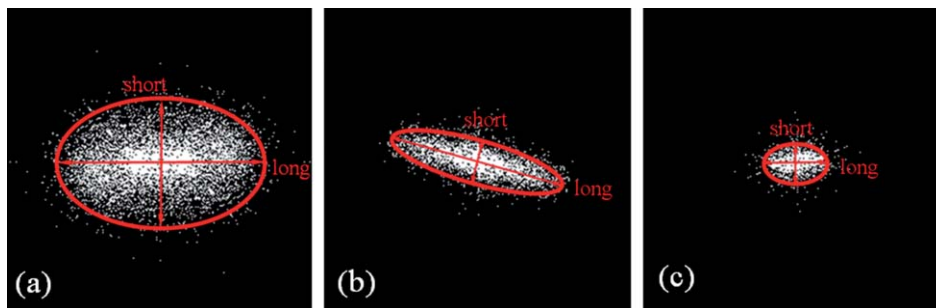

Fig. 2 Power plot of FFT for different aging collagen. (a) 8 weeks of chronological aging; (b) 60 weeks of chronological aging; (c) photoaging.
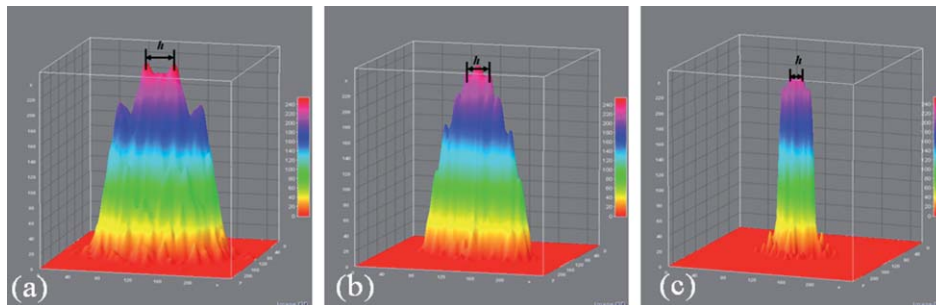

Fig. 3 3D images of different statuses of aging. (a) 8 weeks; (b) 60 weeks; (c) photoaging skin.

Table 1 Quantitative analysis of the collagen orientation index and collagen bundle packing with ages.

Skin situation	8 weeks	16 weeks	50 weeks	60 weeks	photo-aging
collagen orientation index (N)	0.32 ± 0.02	0.45 ± 0.05^a	0.71 ± 0.03^a	0.78 ± 0.02^a	0.03 ± 0.03^a
collagen bundle packing (L)	11.8 ± 0.8^a	13.5 ± 0.7^a	18.1 ± 0.7^a	18.5 ± 0.9^a	25.3 ± 0.9^a

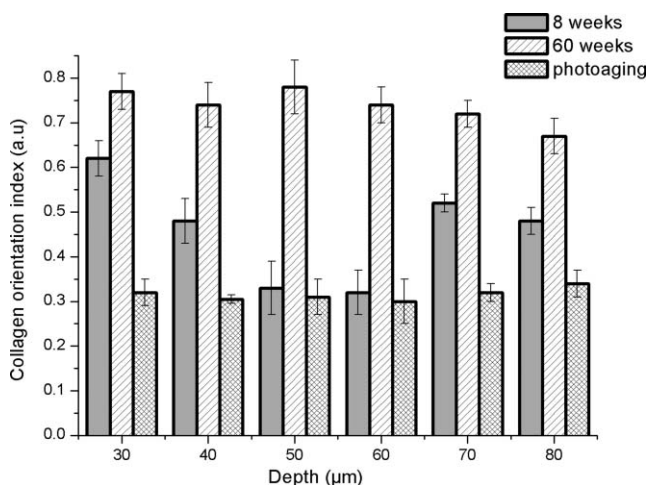Values are means $\pm$ SD, statistic difference value (*P*): a means obvious difference (P < 0.05).

Fig. 4 Collagen orientation index with detectable depth of aging skin.

detectable depth and it is the smallest. These results indicate that the collagen alignment is considerably parallel in all layers and the number of collagen fibrils reduces for chronological aging skin. For photoaging skin, the collagen fibrils were destroyed by UV irradiation, resulting in the slight change of *N* value with depths (Fig. 4).

4 Conclusion

The texture of chronological aging and photoaging of mice skin are characterized and quantitatively determined *in vivo* based on multiphoton microscopy in combination with fast Fourier transform. Our study suggests that the collagen-related characteristics of orientation index and bundle packing are good indicators for the types of aging skin and their exact statuses of aging processes. In addition, the factors resulting in skin damages may be visualized. These results are helpful for the improvement and monitoring of aging skin treatment in the near future.

References

1. A. B. Cua, K. P. Wilhelm, and H. I. Maibach, "Elastic properties of human skin: relation to age, sex, and anatomical region," *Arch. Dermatol. Res.* **282**, 283–288 (1990).
2. Y. R. Helfrich, D. L. Sachs, and J. J. Voorhees, "Overview of skin aging and photoaging," *Dermatol. Nurs.* **20**, 177–183 (2008).
3. M. Domyati, S. Attia, F. Saleh, D. Brown, D. E. Birk, F. Gasparro, H. Ahmad, and J. Utto, "Intrinsic aging versus photoaging: a comparative histopathological, immunohistochemical, and ultrastructural study of skin," *Exp. Dermatol.* **11**, 398–405 (2002).
4. A. J. Bailey, "Molecular mechanisms of aging in connective tissue," *Mech. Ageing Dev.* **122**, 735–755 (2001).
5. F. M. Hendriks, D. Brokken, C. W. J. Oomens, D. L. Bader, and F. P. T. Baaijens, "The relative contributions of different skin layers to the mechanical behavior of human skin *in vivo* using suction experiments," *Med. Eng. Phys.* **28**, 259–266 (2006).
6. W. Denk, J. H. Strickler, and W. W. Webb, "Two-photon laser scanning fluorescence microscopy," *Science* **248**, 73–76 (1990).
7. R. Gauderon, P. B. Lukins, and J. R. Sheppard, "Simultaneous multi-channel nonlinear imaging: combined two-photon excited fluorescence and second-harmonic generation microscopy," *Micron*. **32**, 685–689 (2001).
8. V. R. Korde, G. T. Bonnema, W. Xu, C. Krishnamurthy, J. R. Moore, K. Saboda, L. D. Slayton, S. J. Salasche, J. A. Warneke, D. S. Alberts, and J. K. Barton, "Using optical coherence tomography to evaluate skin sun damage and precancer," *Lasers Surg. Med.* **39**, 687–695 (2007).
9. S. Neerken, G. Lucassen, M. Bisschop, E. Lenderink, and T. A. M. Nuijs, "Characterization of age-related effects in human skin: a comparative study that applies confocal laser scanning microscopy and optical coherence tomography," *J. Biomed. Opt.* **9**, 274–281 (2004).
10. A. L. Branzan, M. Landthaler, and R. M. Szeimies, "*In vivo* confocal scanning laser microscopy in dermatology," *Lasers Med. Sci.* **22**, 73–82 (2007).
11. Y. Miyamae, Y. Yamakawa, M. Kawabata, and Y. Ozaki, "A noninvasive method for assessing interior skin damage caused by chronological aging and photoaging based on near-infrared diffuse reflection spectroscopy," *Appl. Spectrosc.* **62**, 677–681 (2008).
12. S. Zhuo, J. Chen, S. Xie, Z. Hong, and X. Jiang, "Extracting diagnostic stromal organization features based on intrinsic two-photon excited fluorescence and second-harmonic generation signals," *J. Biomed. Opt.* **14**, 020503 (2009).
13. S. Yen, W. Chen, S. Lin, S. Jee, Y. Chen, L. Lin, P. T. C. So, and C. Dong, "Investigating mechanisms of collagen thermal denaturation by high resolution second-harmonic generation imaging," *Biophys. J.* **91**, 2620–2625 (2006).
14. M. J. Koehler, K. König, P. Elsner, R. Bückle, and M. Kaatz, "*In vivo* assessment of human skin aging by multiphoton laser scanning tomography," *Opt. Lett.* **31**, 2879–2881 (2006).
15. R. Cicchi, D. Kapsokalyvas1, V. De Giorgi, V. Maio, A. Van Wiechen, D. Massi, T. Lotti, and F. S. Pavone, "Scoring of collagen organization in healthy and diseased human dermis by multiphoton microscopy," *J. Biophoton.* **3**, 34–43 (2010).
16. K. Lu, J. Chen, S. Zhuo, L. Zheng, X. Jiang, X. Zhu, and J. Zhao, "Multiphoton laser scanning microscopy of localized scleroderma," *Skin Res. Technol.* **15**, 489–495 (2009).
17. P. P. M. van Zuijlen, J. J. B. Ruurda, H. A. van Veen, J. van Marle, A. J. M. van Trier, F. Groenevelt, R. W. Kreis, and E. Middelkoop, "Collagen morphology in human skin and scar tissue: no adaptations in response to mechanical loading at joints," *Burns.* **29**, 423–431 (2003).
18. H. J. de Vries, D. N. Enomoto, J. van Marle, P. P. M. van Zuijlen, J. R. Mekkes, and J. D. Bos, "Dermal organization in scleroderma: the Fast Fourier Transform and the laser scatter method objectify fibrosis in non-lesional as well as lesional skin," *Lab. Invest.* **80**, 1281–1289 (2000).