Correlation between polarization sensitive optical coherence tomography and second harmonic generation microscopy in skin

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Abstract: Both polarization sensitive optical coherence tomography (PS-OCT) and second harmonic generation (SHG) microscopy are 3D optical imaging methods providing information related to collagen in the skin. PS-OCT provides birefringence information which is due to the collagen composition of the skin. SHG microscopy visualizes collagen fibers in the skin based on their SHG property. These two modalities have been applied to the same skin pathologies associated with collagen changes, but their relationship has not been examined. In this study, we tried to find the relationship by imaging the same skin samples with both modalities. Various parts of the normal rat skin and burn damaged skin were imaged ex vivo, and their images were analyzed both qualitatively and quantitatively. PS-OCT images were analyzed to obtain tissue birefringence. SHG images were analyzed to obtain collagen orientation indices by applying 2D Fourier transform. The skin samples having higher birefringence values had higher collagen orientation indices, and a linear correlation was found between them. Burn damaged skin showed decreases in both parameters compared to the control skins. This relationship between the bulk and microscopic properties of skin may be useful for further skin studies.

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References and links

1. J. F. de Boer and T. E. Milner, “Review of polarization sensitive optical coherence tomography and Stokes vector determination,” J. Biomed. Opt. 7(3), 359–371 (2002).
2. K. H. Kim, B. H. Park, Y. Tu, T. Hasan, B. Lee, J. Li, and J. F. de Boer, “Polarization-sensitive optical frequency domain imaging based on unpolarized light,” Opt. Express 19(2), 552–561 (2011).
3. J. F. de Boer, T. E. Milner, M. J. C. van Gemert, and J. S. Nelson, “Two-dimensional birefringence imaging in biological tissue by polarization-sensitive optical coherence tomography,” Opt. Lett. 22(12), 934–936 (1997).
4. K. H. Kim, M. C. Pierce, G. Maguluri, B. H. Park, S. J. Yoon, M. Lydon, R. Sheridan, and J. F. de Boer, “In vivo imaging of human burn injuries with polarization-sensitive optical coherence tomography,” J. Biomed. Opt. 17(6), 066012 (2012).
5. B. H. Park, C. Saxer, S. M. Srinivas, J. S. Nelson, and J. F. de Boer, “In vivo burn depth determination by high-speed fiber-based polarization sensitive optical coherence tomography,” J. Biomed. Opt. 6(4), 474–479 (2001).
6. M. C. Pierce, R. L. Sheridan, B. Hyle Park, B. Cense, and J. F. de Boer, “Collagen denaturation can be quantified in burned human skin using polarization-sensitive optical coherence tomography,” Burns 30(6), 511–517 (2004).
7. S. M. Srinivas, J. F. de Boer, H. Park, K. Keikhanzadeh, H. E. Huang, J. Zhang, W. O. Jung, Z. Chen, and J. S. Nelson, “Determination of burn depth by polarization-sensitive optical coherence tomography,” J. Biomed. Opt. 9(1), 207–212 (2004).
8. S. Sakai, N. Nakagawa, M. Yamamori, A. Miyazawa, Y. Yasuno, and M. Matsumoto, “Relationship between dermal birefringence and the skin surface roughness of photoaged human skin,” J. Biomed. Opt. 14(4), 044032 (2009).
9. S. Sakai, M. Yamamari, A. Miyazawa, M. Matsumoto, N. Takagawa, T. Sugawara, K. Kawabata, T. Yatagai, and Y. Yasuno, “In vivo three-dimensional birefringence analysis shows collagen differences between young and old photo-aged human skin,” J. Invest. Dermatol. 128(7), 1641–1647 (2008).

10. P. J. Campagnola and L. M. Loew, “Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms,” Nat. Biotechnol. 21(11), 1356–1360 (2003).

11. T. Yasui, M. Yonesu, R. Tanaka, Y. Tanaka, S. Fukushima, T. Yamashita, Y. Ogura, T. Hirao, H. Murota, and T. Araki, “In vivo observation of age-related structural changes of dermal collagen in human facial skin using collagen-sensitive second harmonic generation microscope equipped with 1250-nm mode-locked Cr:Forsterite laser,” J. Biomed. Opt. 18(3), 031108 (2013).

12. S. Zhou, X. Zhu, J. Chen, and S. Xie, “Quantitative biomarkers of human skin photoaging based on intrinsic second harmonic generation signal,” Scanning 35(4), 273–276 (2013).

13. Y.-H. Liao, W.-C. Kuo, S.-Y. Chou, C.-S. Tsai, G.-L. Lin, M.-R. Tsai, Y.-T. Shih, G.-G. Lee, and C.-K. Sun, “Quantitative analysis of intrinsic skin aging in dermal papillae by in vivo harmonic generation microscopy,” Biomed. Opt. Express 5(9), 3266–3279 (2014).

14. S. J. Lin, S. H. Jee, C. J. Kuo, R. J. Wu, W. C. Lin, J. S. Chen, Y. H. Liao, C. J. Hsu, T. F. Tsai, Y. F. Chen, and C. Y. Dong, “Discrimination of basal cell carcinoma from normal dermal stroma by quantitative multiphoton imaging,” Opt. Lett. 31(18), 2756–2758 (2006).

15. R. LaComb, O. Nadiarnykh, and P. J. Campagnola, “Quantitative Second Harmonic Generation Imaging of the Diseased State Osteogenesis Imperfecta: Experiment and Simulation,” Biophys. J. 94(11), 4504–4514 (2008).

16. R. Tanaka, S. Fukushima, K. Sasaki, Y. Tanaka, H. Murota, T. Matsumoto, T. Araki, and T. Yasui, “In vivo visualization of dermal collagen fiber in skin burn by collagen-sensitive second-harmonic-generation microscopy,” J. Biomed. Opt. 18(6), 061231 (2013).

17. S. L. Wu, H. Li, X. M. Zhang, W. R. Chen, and Y. X. Wang, “Character of skin on photo-thermal response and its regeneration process using second-harmonic generation microscopy,” Lasers Med. Sci. 29(1), 141–146 (2014).

18. S.-Y. Chen, C. Shee-Uan, W. Hai-Yin, L. Wen-Jeng, Y.-H. Liao, and C.-K. Sun, “In vivo virtual biopsy of human skin by using noninvasive higher harmonic generation microscopy,” IEEE J. Sel. Top. Quantum Electron. 16(3), 478–492 (2010).

19. N. Vogler, A. Medyukhina, I. Latka, S. Kemper, M. Böhm, B. Dietzek, and J. Popp, “Towards multimodal nonlinear optical tomography – experimental methodology,” Laser Photon. Lett. 8(8), 617–624 (2011).

20. M.-G. Lin, T.-L. Yang, C.-T. Chiang, H.-C. Kao, J.-N. Lee, W. Lo, S.-H. Jee, Y.-F. Chen, C.-Y. Dong, and S.-J. Lin, “Evaluation of dermal thermal damage by multiphoton autofluorescence and second-harmonic-generation microscopy,” J. Biomed. Opt. 11(6), 064006 (2006).

21. P. Matteini, F. Ratto, F. Rossi, R. Cicchi, C. Stringari, D. Kapsokalyvas, F. S. Pavone, and R. Pini, “Photothermally-induced disordered patterns of corneal collagen revealed by SHG imaging,” Opt. Express 17(6), 4868–4878 (2009).

22. S. Wu, H. Li, H. Yang, X. Zhang, Z. Li, and S. Xu, “Quantitative analysis on collagen morphology in aging skin based on multiphoton microscopy,” J. Biomed. Opt. 16(4), 040502 (2011).

23. R. M. Aspden, Y. E. Yarker, and D. W. Hukins, “Collagen orientations in the meniscus of the knee joint,” J. Anat. 140(3), 371–380 (1985).

24. A. Aparecida de Aro, B. C. Vidal, and E. R. Pinentel, “Biochemical and anisotropical properties of tendons,” Micron 43(2-3), 205–214 (2012).

25. H. J. C. de Vries, D. N. Enomoto, J. van Marle, P. P. van Zuilen, J. R. Mekkes, and J. D. Bos, “Dermal organization in Scleroderma: The Fast Fourier Transform and the laser scatter method objectify fibrosis in nonlesional as well as lesional skin,” Lab. Invest. 80(8), 1281–1289 (2000).

26. J. Brasselet, “Polarization-resolved nonlinear microscopy: application to structural molecular and biological imaging,” J. Opt. Photon. 3(3), 205–271 (2011).

27. T. Yasui, K. Sasaki, Y. Tohno, and T. Araki, “Tomographic Imaging of Collagen Fiber Orientation in Human Tissue Using Depth-Resolved Polarimetry of Second-Harmonic-Generation Light,” Opt. Quantum Electron. 37(13-15), 1397–1408 (2005).

28. V. A. Hovhannisyan, P.-S. Hu, H.-Y. Tan, S.-J. Chen, and C.-Y. Dong, “Spatial orientation mapping of fibers using polarization-sensitive second harmonic generation microscopy,” J. Biophotonics 5(10), 768–776 (2012).

29. A. Erikson, J. Ortgren, T. Hompland, C. de Lange Davies, and M. Lindgren, “Quantification of the second-order nonlinear susceptibility of collagen I using a laser scanning microscope,” J. Biomed. Opt. 12(4), 044002 (2007).

30. S. Psolomidoupolous, V. Petegnief, G. Soria, I. Attari-Roldan, D. Artigas, A. M. Planas, and P. Loza-Alvarez, “Estimation of the effective orientation of the SHG source in primary cortical neurons,” J. Biomed. Opt. 17(16), 14414–14425 (2009).

31. B. Baumann, W. Choi, B. Potsaid, D. Huang, J. S. Duker, and J. G. Fujimoto, “Swept source/Fourier domain polarization sensitive optical coherence tomography with a passive polarization delay unit,” Opt. Express 20(9), 10229–10241 (2012).

32. B. Jeong, B. Lee, M. S. Jang, H. Nam, S. J. Yoon, T. Wang, J. Doh, B.-G. Yang, M. H. Jang, and K. H. Kim, “Combined two-photon microscopy and optical coherence tomography using individually optimized sources,” Opt. Express 19(14), 13089–13096 (2011).

33. X. Chen, O. Nadiarnykh, S. Plotnikov, and P. J. Campagnola, “Second harmonic generation microscopy for quantitative analysis of collagen fibrillar structure,” Nat. Protoc. 7(4), 654–669 (2012).

34. E. F. Bernstein, Y. Q. Chen, J. B. Kopp, L. Fisher, D. B. Brown, P. J. Hahn, F. A. Robey, J. Lakkakorpi, and J. Uitto, “Long-term sun exposure alters the collagen of the papillary dermis. Comparison of sun-
protected and photoaged skin by Northern analysis, immunohistochemical staining, and confocal laser scanning microscopy,” J. Am. Acad. Dermatol. 34(2 Pt 1), 209–218 (1996).

35. S. P. Chong, T. Lai, Y. Zhou, and S. Tang, “Tri-modal microscopy with multiphoton and optical coherence microscopy/tomography for multi-scale and multi-contrast imaging,” Biomed. Opt. Express 4(9), 1584–1594 (2013).

36. S. Tang, Y. Zhou, K. K. H. Chan, and T. Lai, “Multiscale multimodal imaging with multiphoton microscopy and optical coherence tomography,” Opt. Lett. 36(24), 4800–4802 (2011).

37. G. Liu and Z. Chen, “Fiber-based combined optical coherence and multiphoton endomicroscopy,” J. Biomed. Opt. 16(3), 036010 (2011).

38. J. Xi, Y. Chen, Y. Zhang, K. Murari, M.-J. Li, and X. Li, “Integrated multimodal endomicroscopy platform for simultaneous en face optical coherence and two-photon fluorescence imaging,” Opt. Lett. 37(3), 362–364 (2012).

1. Introduction

Skin is the outer covering of the body and plays a key role in protecting against influences of the environment. Skin has layered structures consisting of the epidermis, dermis, and hypodermis from superficial to deep. The superficial epidermis consists of cells that form the protecting layer. The underlying dermis consists mainly of extracellular matrix (ECM) components such as collagen and elastin, and contributes to the strength and toughness of the skin. Since skin diseases are accompanied by structural alternation, visualization of skin structures would significantly facilitate skin disease diagnosis and treatment. Various optical imaging techniques have been applied to skin studies such as polarization sensitive optical coherence tomography (PS-OCT) and second harmonic generation (SHG) microscopy which are promising methods for studying the skin diseases related to collagen changes.

PS-OCT is an augmented form of OCT and provides information of both structure and birefringence by measuring polarization as well as intensity of reflected light from within tissues [1–3]. Birefringence is an optical property indicating anisotropy of refractive index depending on polarization. Skin is birefringent due to collagen composition in the dermis. PS-OCT has been applied to various skin studies associated with collagen changes such as skin burn [4–7] and photo-aging [8, 9]. SHG microscopy is a microscopic technique based on a nonlinear SHG effect: emission photons are generated by combining two excitation photons at the focus of excitation light and emission light has half the wavelength of excitation light. Collagen has SHG property due to its non-centrosymmetric molecular structure [10]. SHG microscopy visualizes collagen fibers in the skin dermis and has been applied to various skin studies such as skin aging [11–13], skin cancer [14], Osteogenesis Imperfecta [15], and skin burn [16, 17]. SHG microscopy has also been applied to in vivo human skin studies [11, 13, 18]. SHG microscopy is often in combination with two-photon microscopy by sharing the light source for both nonlinear two-photon excitation and SHG. Multimodal approaches combining SHG microscopy with two-photon excited fluorescence, third harmonic generation and coherence anti-Stokes Raman scattering microscopy were developed [19]. For SHG image analysis, SHG intensity, organization, and complexity of collagen fiber distribution have been used as quantitative parameters [16, 17, 20–22]. SHG intensity based analysis methods often suffer from thickness variation of the skin epidermis. Collagen organization is important because it is related to mechanical properties and function of the skin [23, 24]. Collagen organization was characterized by collagen orientation index which could be obtained from SHG images by either Fast Fourier Transform (FFT) or laser scattering method [17, 22, 25]. Recently, polarization sensitive SHG (PS-SHG) microscopy, which utilizes polarization sensitivity of SHG intensity from collagen fibers and other SHG source, has been developed to provide more detailed information [21, 26–30]. Measurement of 3D orientation and structural anisotropy of collagen fibers in various tissues has been demonstrated.

Since both PS-OCT and SHG microscopy provide information about collagen, there must be some relationship between them. In this study, we tried to bridge between the two modalities. Various sites of the normal rat skin such as the back, abdomen, and rear leg and burn damaged skin were imaged by both PS-OCT and SHG microscopy.
Quantitative analysis was performed to obtain tissue birefringence and collagen orientation index. Correlation between the two parameters was analyzed statistically.

2. Materials and methods

2.1 Sample preparation

All experiments were performed by using Sprague-Dawley rats (150-200g), which were bred at the animal facility of POSTECH Biotech Center under specific pathogen-free conditions. Rat hair was removed using a depilatory after the rats were anesthetized with isoflurane. Then, the rats were sacrificed by cervical dislocation and full thickness skin samples of approximately 2 cm x 2 cm in size were excised from the back, abdomen, and rear leg. For burn skin samples, the rats were anesthetized and the burn was induced on the left rear leg by applying 85 °C water for 10 seconds one day after removing the hair. One hour after the burn, the rats were sacrificed by cervical dislocation and the burn damaged skins were excised with full skin thickness. The skins of the right rear legs were taken as the contralateral control samples. Excised skin samples were flattened and fixed in formaldehyde (4%) for 1 h, washed and preserved in phosphate buffered saline (PBS) solution at 4 °C, and used for experiments within one week. These skin samples were imaged by both PS-OCT and SHG microscopy. After imaging, the skin samples were sectioned with thickness of 10 μm and then stained using Masson’s trichrome (MT) for histological analysis of dermis collagen.

2.2 Imaging system

For PS-OCT imaging, a custom PS-OCT system based on passive polarization delay unit (PDU) [31] was used. In short, the system used a wavelength swept-source (SSOCT-1310, AXSUN Technologies), which had center wavelength of 1310 nm, bandwidth of 107 nm, sweeping speed of 50 kHz and imaging depth range of 6 mm in the air. Illumination light was generated by PDU, it consisted of two orthogonal polarization states with optical path separation by 2.25 mm from each other. Reflection of the two polarization states from the sample was collected in single acquisition at different depths. Light from the source was split with a 95:5 ratio; 95% of light went to the interferometry setup for OCT, and 5% went to a fiber Bragg grating (FBG) to generate an external trigger signal for data acquisition. In the interferometer, light was split into the sample and reference arms with an 80:20 ratio respectively by a fiber coupler. Light in the sample arm passed through the PDU, a 2D galvano mirror scanner, an objective lens (LSM03, Thorlabs), and then was focused at the sample. Light in the reference arm passed through a delay line in order to match optical path lengths in both arms. Reflected light from the sample was combined with reflected light from the reference arms at the detection arm, and interference was collected by two balanced photodetectors (BDP410C, Thorlabs) in the polarization diverse detection setup. 1280 samples per depth-scan were acquired by a two-channel data acquisition board (Alazartech). Data was post-processed to get both intensity and PS images. Dispersion difference between the reference and sample arms was compensated numerically by using pre-calibration data of a mirror sample. Simultaneous sample illumination with two polarization states enabled determination of the depth-resolved Jones matrices of the sample. Polarization properties of the sample were obtained by analyzing the sample Jones matrices through eigenvector decomposition [2, 5]. The signal-to-noise ratio of our system is 103 dB. PS-OCT images consisting of 500 pixels x 500 pixels in the x-y plane were taken with the lateral scan over 5 mm x 5 mm, and the imaging time was 6 seconds for each volume scan. Each skin sample was imaged at three random sites.

For SHG imaging, a conventional SHG microscope with circular polarization illumination were used to obtain SHG images of the collagen within skin. The microscope configuration was similar to the custom built two-photon laser scanning microscope that was used in a previous study [32]. In brief, a Ti-Sapphire laser (Chameleon Ultra II, Coherent), which had 140-fs pulse width and 80-MHz pulse repetition rate, was used as the light source. The laser beam from the source first passed through the combination of a half-wave plate and a polarizer for power control, and
through a quarter-wave plate (WPQ05M-850) in order to convert the polarization of light from linear to circular. This was to avoid the polarization dependency of SHG signal [33]. The laser beam reflected on a pair of galvanometric scanners (6215H, Cambridge Technology) for the raster scanning, passed through a pair of scan and tube lenses for beam expansion, and then went into an upright microscope (BX51, Olympus). The laser beam passed through a dichroic mirror (DM1, 1025DCSP, Chroma, a 20x objective lens (XLUMPLFLN, NA 1, Olympus) and was focused on the sample. SHG light generated in the sample was collected back by the objective lens, reflected on the dichroic mirror, passed through a combination of a barrier filter and an emission filter, and was collected by a photomultiplier tube (H7421-40, Hamamatsu) in the photon count mode. The signal was processed and image was displayed in real time by a customized LabVIEW program.

The excitation wavelength was selected to be 790 nm, and SHG light was filtered in the detection by using a 400 nm band-pass filter (HQ 400/20M-2P, Chroma). The imaging field of view was 300 µm x 300 µm in the x-y plane with 512 pixels x 512 pixels and the power at the objective lens back aperture was adjusted to be approximately 25 mW. The acquisition time was about 15 seconds per frame. Each skin sample was imaged five times at different sites.

2.3 Quantitative analysis method

PS-OCT images of the skin were analyzed to obtain tissue birefringence, which was calculated as the slope of the accumulated phase retardation with depth [2–4]. For each cross-sectional PS-OCT, the accumulated phase retardation was averaged over the full width of the image in the x-z plane and was plotted as a function of depth. The depth range of interest in the plot was set to be from the surface to approximately 400 µm deep. The weighted linear least squares regression was applied to the first linear region of the plot for slope estimation.

SHG images of the skin were analyzed to characterize the anisotropy of collagen fiber orientation by using 2D FFT. Each 3D SHG image stack was first processed to obtain the depth projection image. From the depth projection image, FFT was applied and the FFT power spectrum was plotted, then an ellipse curve fitting was applied to the power plot. The collagen orientation index I was calculated as $I = 1 - S/L$ [17, 22], where S and L were the lengths of minor and major axes of the fitted ellipse respectively. The value of I increases as the orientation of the structure increases. In contrast, if the structure is randomly organized, i.e., isotropic, $I = 0$ and the FFT plot image becomes circular. For statistical analysis, correlation analysis was performed from data of fourteen skin samples.

3. Results

3.1 PS-OCT images of the rat skin

PS-OCT images of two different sites of the rat skin ex vivo are shown in Fig. 1. OCT intensity and polarization-sensitive (PS) images of the rear leg skin and back skin are shown in Fig. 1(a)-1(b) and Fig. 1(c)-1(d) respectively, and the plots of the average accumulated phase retardations with depth are shown in Fig. 1(e). OCT intensity images (Fig. 1(a), 1(c)) of both skin sites showed skin structure from the surface down to the subcutaneous fat layer. The superficial epidermis was not distinguishable from the dermis in both skin sites because the epidermis was too thin. The dermis was clearly distinguished from the subcutaneous fat layer due to differences in scattering property. The dermis of leg skin was much thinner than that of the back skin. PS images (Fig. 1(b), 1(d)) showed accumulated phase retardation with depth from the surface in a gray scale where black and white corresponded to 0° and 180° respectively. Further accumulation of phase retardation wrapped around back to black color. PS images clearly showed black-to-white color transition patterns with depth indicating birefringence in both skin sites. PS image of the rear leg skin showed the black-to-white transition within the shorter distance compared to the one of the back skin. The accumulated phase retardation of each PS-OCT image was averaged over the full width of the image and plotted as a function of depth (Fig. 1(e)). The initial linear region in the accumulated phase retardation plot of the...
leg skin was steeper than the one of the back skin. At 150 μm depth, the phase retardation reached 115° in the leg skin and 70° in the back skin. The slopes in the linear regions were 0.55°μm−1 and 0.34°μm−1 in the presented rear leg skin and back skin respectively. These plots showed that the rear leg skin had higher birefringence than the back skin.

3.2 SHG images of the rat skin

SHG images of the rear leg skin and back skin specimens at several depths are shown in Fig. 2(a)-2(d) and Fig. 2(e)-2(h) respectively, and 2D FFT analysis of their projected SHG images are shown in Fig. 2(i) and Fig. 2(j) respectively. SHG images visualized distribution and orientation of collagen fibers at different depths of the skin dermis. SHG images of the leg skin in Fig. 2(a)-2(d) had higher intensities than those of the back skin in Fig. 2(e)-2(h). SHG images of the leg skin sample showed collagen fibers aligned in the vertical direction, and this orientation was consistent throughout the imaging depth. In contrast, SHG images of the back skin sample showed random orientation of collagen fibers. The difference in collagen organization between the two skin sites was shown in 2D FFT power plots in Fig. 2(i)-2(j). The FFT power plot of the rear leg skin sample showed an elliptical shape with a long axis in the horizontal direction due to higher spatial frequency components, whereas the one of the back skin sample showed a relatively circular shape which indicated the random orientation of the collagen fibers. Quantitative analysis showed that the collagen orientation indices of the presented rear leg and back skin were 0.55 and 0.27 respectively.
3.3 Histological images with Masson’s trichrome staining

Histological images of the rear leg skin and back skin samples are shown in Fig. 3(a) and Fig. 3(b) respectively. These histological images were labeled by MT staining to visualize collagen in the dermis. Collagen fibers and cells appeared in blue and red respectively. These histological images showed detailed structures of the skin such as the thin superficial epidermis, collagen fibers and capillary vessels in the dermis. The leg skin had a thinner epidermis than that of the back skin, and both skin samples had comparable collagen fiber density. In addition, the leg skin histological image showed collagen fibers aligned mostly along the image plane while that of the back skin showed fibers aligned perpendicular to the image plane as well. This indicates that the back skin collagen fibers were less organized than the leg skin collagen fibers. This result was consistent with data from the SHG images.

3.4 Correlation analysis between phase retardation slope and collagen orientation index

Correlation between the accumulated phase retardation slopes and collagen orientation indices obtained from various normal skin samples were analyzed. Values of the two properties obtained from fourteen skin samples were plotted in Fig. 4. The phase retardation slopes and collagen orientation indices were plotted in the x and y axes respectively. Correlation analysis was performed between the two properties, and the Pearson’s correlation coefficient was calculated to be 0.86. Since this coefficient was higher than the critical value (0.78) based on fourteen samples for determining a linear correlation, the accumulated phase retardation slope and collagen orientation index were determined to be linearly correlated.
Fig. 4. Correlation between phase retardation slope (x) and collagen orientation index (y) of rat skin. Regression: \( y = 0.92x + 0.12, r^2 = 0.725. \) \( p < 0.001. \)

3.5 PS-OCT and SHG images of the burn damaged rat skin

PS-OCT and SHG microscopy were applied to the burn damaged rat leg skin samples, and their birefringence and collagen orientation indices were analyzed in comparison with those of the contralateral control skin samples. Results of PS-OCT and SHG microscopy are shown in Fig. 5. Intensity and PS images of the contralateral control skin and burn damaged skin are shown in Fig. 5(a)-5(b) and 5(c)-5(d) respectively. SHG images of the contralateral control skin and burn damaged skin at different depth locations are shown in Fig. 5(e)-5(h) and 5(i)-5(l) respectively. PS-OCT intensity images showed similar structures in the control skin and burn damaged skin. The burn damaged skin was thicker than the control skin because there was an edema layer under the burn damaged skin due to the burn process. PS-OCT PS image of the control skin showed a clear black-white banding pattern indicating birefringence. On the other hand, PS-OCT PS image of the burn damaged skin showed that the white banding pattern appeared at deeper region compared to control skin indicating weaker birefringence. Birefringence was quantified by estimating the phase retardation slopes from PS-OCT images, and the phase retardation slopes were \( 0.44^\circ\mu\text{m}^{-1} \) and \( 0.17^\circ\mu\text{m}^{-1} \) for the control and burn damaged skins respectively. This result indicated that there was a decrease in birefringence in the burn damaged skin.

SHG images of the control skin showed densely distributed and organized fine collagen fibers. Conversely, SHG images of the burn damaged skin showed coarse collagen fibers which were sparsely distributed and randomly oriented. Quantitatively analysis of the collagen orientation using 2D Fourier Transform gave the result that the value of collagen orientation index of control skin sample was 0.59 and burnt skin sample was 0.32. The burn process induced a significant reduction in collagen orientation index.
4. Discussion and conclusion

PS-OCT and SHG microscopy provided collagen information of the skin based on different contrast mechanisms: birefringence based on light back reflection and collagen fiber distribution based on nonlinear SHG. The relationship between these two modalities was analyzed by imaging the same skin samples. Various sites of the rat skin such as the back, abdomen, and leg were imaged ex vivo, and statistical analysis was performed. Birefringence of PS-OCT and collagen orientation index of SHG microscopy were found to be linearly correlated. In addition, the skin burn experiment was conducted. The decrease in both birefringence and collagen orientation index were observed in burn damaged skin samples. This correlation could be explained as follows: birefringence is a cumulative and bulk property, demonstrating its ability to change the polarization state of light. Birefringence in the skin comes mostly from collagen in the dermis. If a skin sample has organized collagen fibers within some thickness, they will induce a cumulative effect on the polarization state of light and the skin becomes birefringent. The linear relationship found in this study is consistent with separate skin studies of photo-aging; the photo-aged skin had smaller birefringence [9], disorganized collagen fibers [34], and decreases in collagen orientation index [22].

From the burn experiment, the birefringence change and collagen structure difference between control and burn damaged skin samples were successfully visualized and quantified with PS-OCT and SHG microscopy. Due to the thermal effect, the dermal
birefringence decreased dramatically and the collagen structure became sparse and less organized. This is consistent with previous burn studies with PS-OCT [4–7] and SHG microscopy [16, 17].

SHG intensity could be used to quantify the collagen content in the skin [16], but it was not applicable in this study: various sites of skin samples were compared and these skin samples had different thickness of the epidermis. Another property that could be related to the birefringence was collagen density, which was calculated as the ratio of the number of pixels with valid SHG signals to the total number of pixels in the image [12]. Since birefringence mostly originated from collagen, the collagen density obviously affected the tissue birefringence. To examine this relationship, the collagen fiber density was analyzed from SHG images. For the control skin, there was no significant difference in collagen fiber density between samples with different birefringence (data not shown). This result was also consistent with MT histological images (Fig. 3). Therefore, only collagen orientation was correlated with tissue birefringence in the control skin samples. However, in the burn experiment result, SHG images of the burn damaged skin showed sparser distribution of collagen fibers compared to those of the control skin. It suggested that the decrease of birefringence in the burn damaged skin was caused not only by the decrease of collagen orientation but also by the decrease of the collagen density.

With the understanding of the correlation between PS-OCT and SHG microscopy in skin imaging, application of these two methods to skin studies might be improved. PS-OCT and SHG microscopy have been already applied separately to skin burn and skin aging studies. PS-OCT has high-speed imaging and large field of view whereas SHG microscopy gives microscopic information at high resolutions. Therefore, combining the two methods together would increase the accuracy of skin assessment. There were some multi-photon microscopy and conventional OCT combined systems have been developed [32, 35–38]. In the next stage, we are planning to develop a combined system that can perform PS-OCT and SHG microscopy together, and to apply the system to further burn studies. We also aim to extend this study to in vivo human skin. In the current study, conventional SHG microscopy with circular polarization illumination was used to analyze the collagen orientation. Recently developed polarization PS-SHG microscopy provides more detail information of collagen fibers by measuring polarization dependent SHG intensity [21, 26–30]. Combination of PS-OCT and PS-SHG microscopy may be more effective tool to study collagen-associated diseases.

In conclusion, correlation between PS-OCT and SHG microscopy in the skin was examined by analyzing their images of the same skin samples both qualitatively and quantitatively. A linear correlation between birefringence and collagen orientation index from PS-OCT and SHG microscopy was found through statistical analysis. This correlation between the macroscopic and microscopic properties of skin dermis might be useful in further studies of skin diseases.

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