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Noninvasive *in situ* Assessment of Structural Alteration of Human Dermis Caused by Photoaging Using a Novel Collagen-Specific Imaging Technique

Toyonobu Yamashita[1], Yuki Ogura[1], Yuji Tanaka[2], Ryosuke Tanaka[2], Takeshi Yasui[2,3], Tsutomu Araki[2], and Tetsuji Hirao[1]

[1]Shiseido Research Center, Yokohama, Kanagawa, Japan

[2]Graduate School of Engineering Science, Osaka University, Toyonaka, Osaka, Japan

[3]Institute of Technology and Science, Tokushima University, Tokushima, Japan

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ABSTRACT

Specific visualization and assessment of human collagen fibers were demonstrated using a developed second-harmonic-generation (SHG) microscope equipped with a Cr:forsterite laser (1250 nm). Initially, we examined the specific sensitivity of SHG imaging for collagen fibers using excised human skin specimens. Age-related dermal alterations in female subjects were then evaluated by means of SHG microscopy. The developed SHG microscope enabled us to observe the collagen fibers in the skin sections; their SHG images correlated well with the Elastica-van-Gieson-stained sections of dermal fibers. In vivo imaging of human skin with a wide field of view was also achieved. En face SHG images of facial skin of female subjects of different ages showed the structural alterations in the collagen fiber network. Additional image analysis of SHG images based on two-dimensional Fourier transformation also reflected age-related alterations. In conclusion, this proposed methodology can be a powerful tool in fields such as anti-aging dermatology and validation of the efficacy of cosmetic products.

INTRODUCTION

Cutaneous photoaging due to long-term exposure to solar ultraviolet radiation causes the degenerative structural alterations in the dermal extracellular matrix, such as the decrease in collagen fibers and the accumulation of abnormal elastic materials, resulting in a loss of skin elasticity and the formation of wrinkles. These age-related alterations in extracellular matrix have been elucidated based on histological observations of the skin specimens. However, further detailed studies on how UV affects the dermal extracellular matrix in healthy subjects during daily life are hampered by the limitation of collecting skin specimens. The development of noninvasive techniques for the assessment of structural alterations of the extracellular matrix is, therefore, an important challenge not only in anti-aging research but also in cosmetology. Recently developed imaging systems applying non-linear optical techniques enabled us to obtain noninvasive optical images of various tissues. Multiphoton microscopy including SHG microscopy allows acquisition of en face sectional images targeted at a specific biological component [1, 2]. SHG microscopy is an attractive technique for imaging of the skin because the SHG light detected by this microscope is specifically generated by collagen molecules in collagen fibers due to structural asymmetry **(Fig. 1)** [3]. In this study, we investigated human dermal structures and its alteration due to UV exposure by means of a novel collagen-specific SHG microscopy system with the aim of understanding photoaging of the human skin.

MATERIALS AND METHODS

Optical imaging systems

We developed a reflection-mode SHG microscope for noninvasive observation of collagen fibers in the human skin **(Fig. 2, 3)** with the aim of overcoming the limitations of commercially available *in vivo* multiphoton microscopes such as

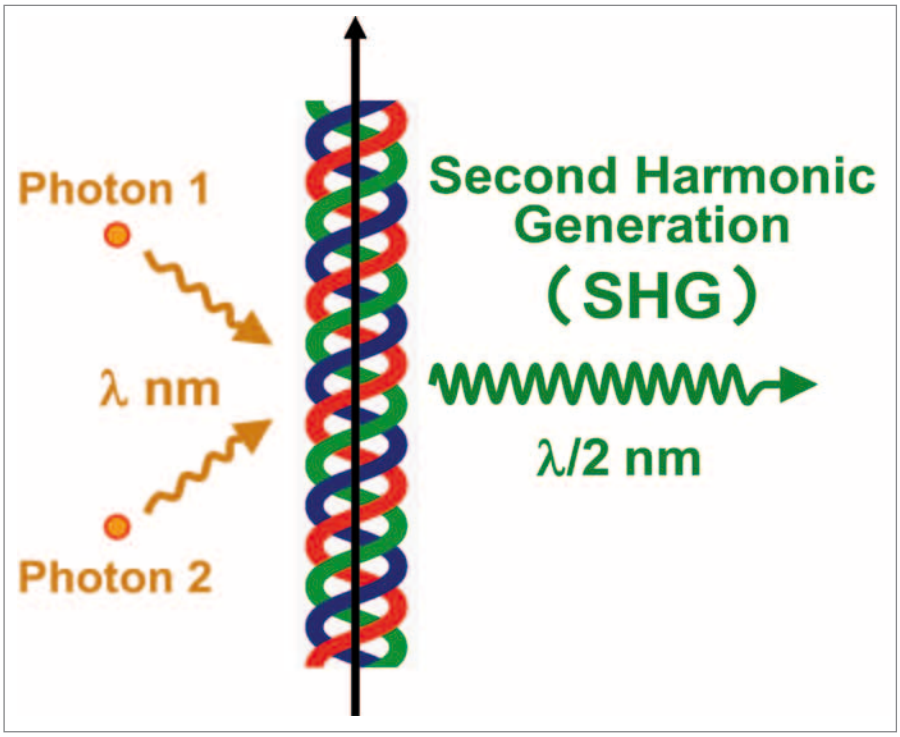

Fig. 1 Theory of SHG emission from collagen fibers. Structurally asymmetric collagen molecules produce SHG light due to simultaneous two-photon absorption when exposed to a femtosecond pulse laser

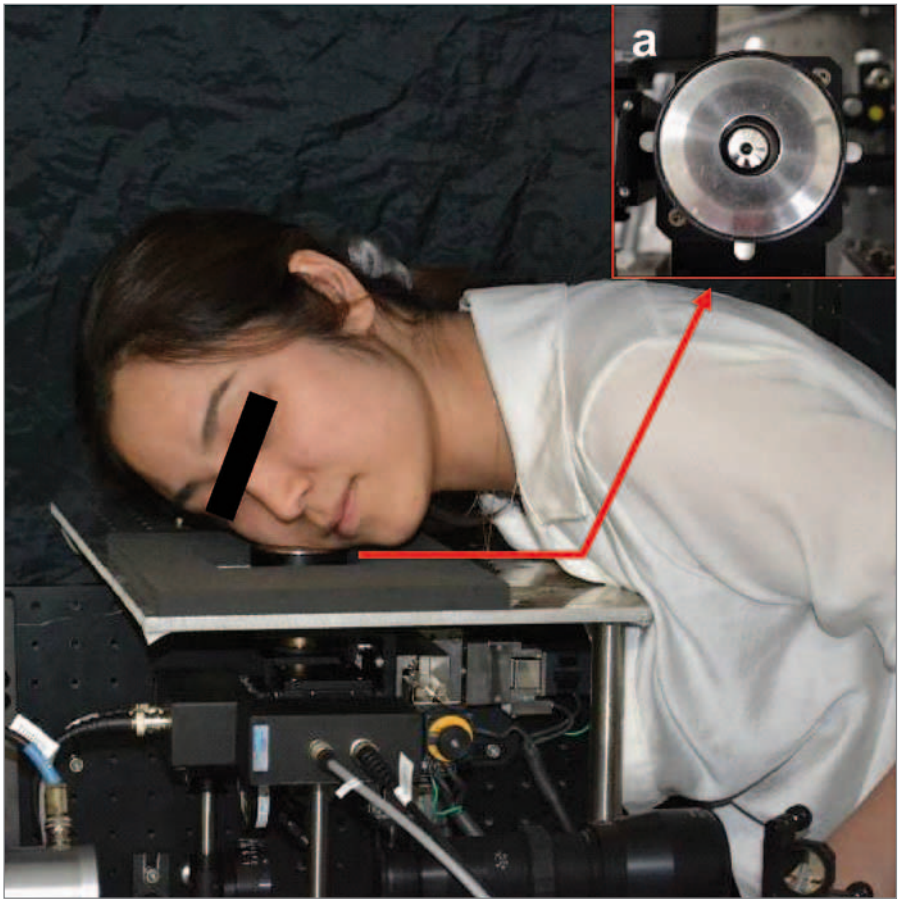

Fig. 2 Photograph of the SHG microscope. (a) 3D-stage

shallow imaging depth, long image acquisition times and small scan areas. This collagen-specific SHG microscope employed as a light source a near infrared femtosecond Cr:forsterite laser (Avesta Project, Moscow, Russia) with a long wavelength (1250 nm). Backscattered SHG light from dermal collagen fibers with a wavelength of 625 nm was detected by a combined photon-counting photomultiplier tube and pulse counter. The 3D stage **(Fig. 2)** used as a mechanical human skin-holding and fixing device was set in this system for acquisition of a wide field of the collagen fiber images.

Specific sensitivity to collagen fibers

Frozen sections of human skin specimens from the face were visualized by SHG microscopy. After SHG imaging, skin sections were stained with the Elastica-van-Gieson method for comparison with the SHG images. These frozen sections of facial skin were obtained from 17- and 64-year-old Asian women.

Evaluation of human skin

The human studies were IRB-approved, and written informed consents were obtained in all cases. Cheek skin was assessed as the sun-exposed site of healthy female subjects aged from the 20s to 50s by means of the SHG microscopy system. Additionally, quantitative image analyses of collagen fibers using Fourier-transformed SHG images were performed, focusing on the density. First, seven regions of interest (each 64 x 64 pixels in size) in the SHG images (1024 x 1024 pixels in size) were selected excluding the area of hair follicles. Then, a Fourier-transformed spectrum of the selected ROI was obtained by two-dimensional Fourier transformation. Finally, the spectrum width (full width at half maximum) was determined as a parameter of collagen density using Gaussian-fitted Fourier-transformed spectrum.

RESULTS AND DISCUSSION

Comparison of SHG images and Elastica-van-Gieson stained section images

SHG images of frozen sections were compared with Elastica-van-Gieson-stained

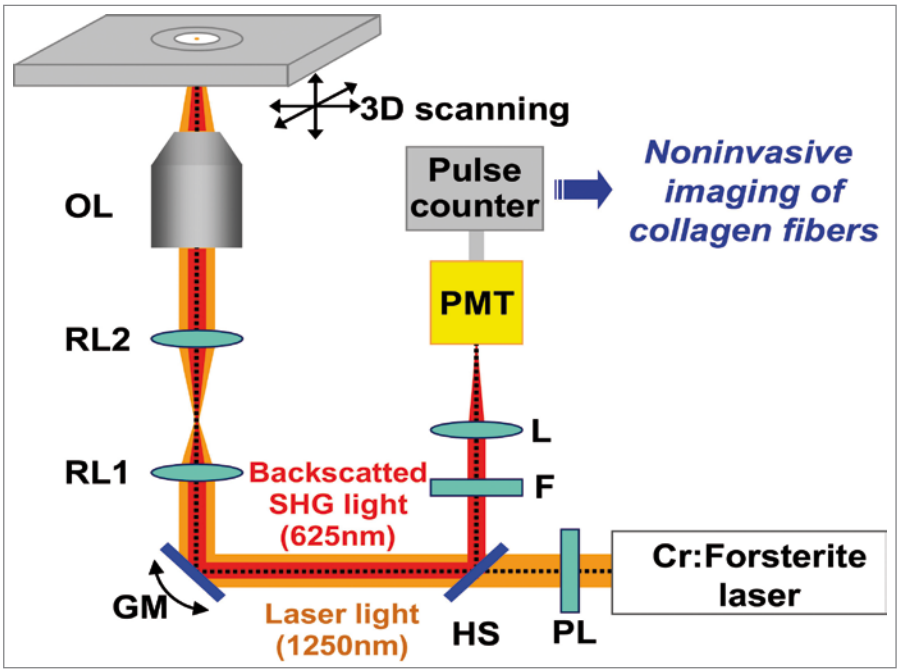

Fig. 3 Experimental setup. PL = polarizer; HS = harmonic separator; F = infrared-cut filter; L = lens, PMT = photomultiplier tube, GM = galvano mirror, RL1 and RL2 = relay lens, OL = oil immersion lens

section images. Representative SHG and stained section images of normal facial skin are shown in Figure 4. As expected, the frozen section of a 17-year-old woman reflected a stronger SHG light than that of a 64-year-old woman **(Fig. 4, a1** versus **b1)**. In addition, the image of collagen fibers in the Elastica-van-Gieson-stained section **(Fig. 4, a2** and **b2)** correlated well with that of the non-stained SHG images **(Fig. 4, a1** and **b1)**. As a characteristic, most of SHG light was observed specifically in the dermis. Also, collagen fibers appearing as a pale-pink-stained color in Elastica-van-Gieson-stained sections showed a strong reflection in the SHG images. We confirmed that bluish-purple-stained elastic fibers resulted in less reflection in SHG images.

In vivo visualization of human cheek skin

Noninvasive visualization of the collagen fiber network in the human reticular dermis was achieved by means of our SHG microscopy system. Highly efficient SHG from collagen fibers due to the Cr:forsterite laser led to an extremely fast acquisition rate of the SHG imaging (600 x 600 µm with 256 x 256 pixels/2 seconds). This fast acquisition rate enabled the acquisition of consecutive en face SHG images of human skin. Moreover, the use of a light source with a longer wavelength (1250 nm) had the advantage of imaging with deeper penetration than with conventional multiphoton microscopy (710-920 nm). Typical depth-resolved SHG images and a wide field SHG image obtained from cheek skin of a healthy male subject in his 60s are shown in Figure 5 and Figure 6, respectively. The SHG images demonstrateed that collagen fiber bundles tend to be aligned parallel to the skin surface. The depth-resolved SHG images showed that the papillary dermis is composed of very tightly packed collagen fiber bundles **(Fig. 5b)** and reticular dermis is composed of bundles that have a larger-diameter **(Fig. 5c** and **d)** than those in the papillary dermis. In addition, a wide field SHG image showed that collagen fibers surrounding many hair follicles were clearly visible as a mesh-like structure in the reticular dermis **(Fig. 6)**.

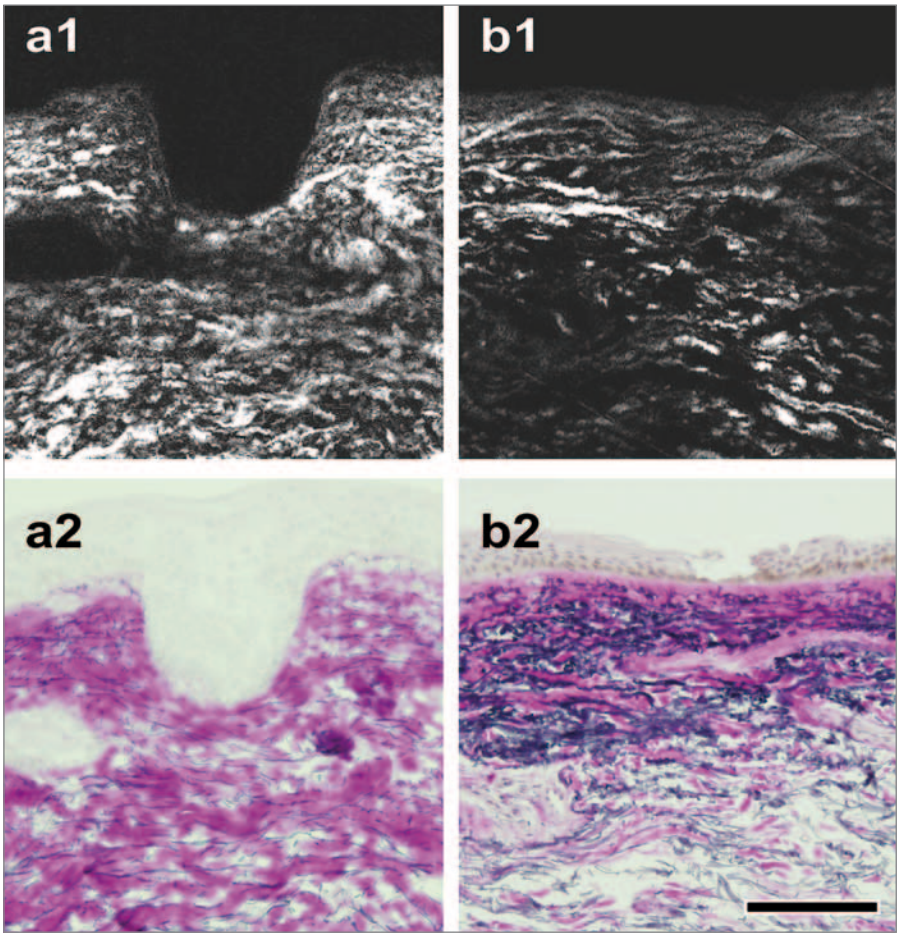

Fig. 4 Frozen section images. (a1 and b1): SHG images, (a2 and b2): Elastica-van-Gieson stained section images, (a1 and a2): 17-year-old woman, (b1 and b2): 64-year-old woman (bars: 200 µm).

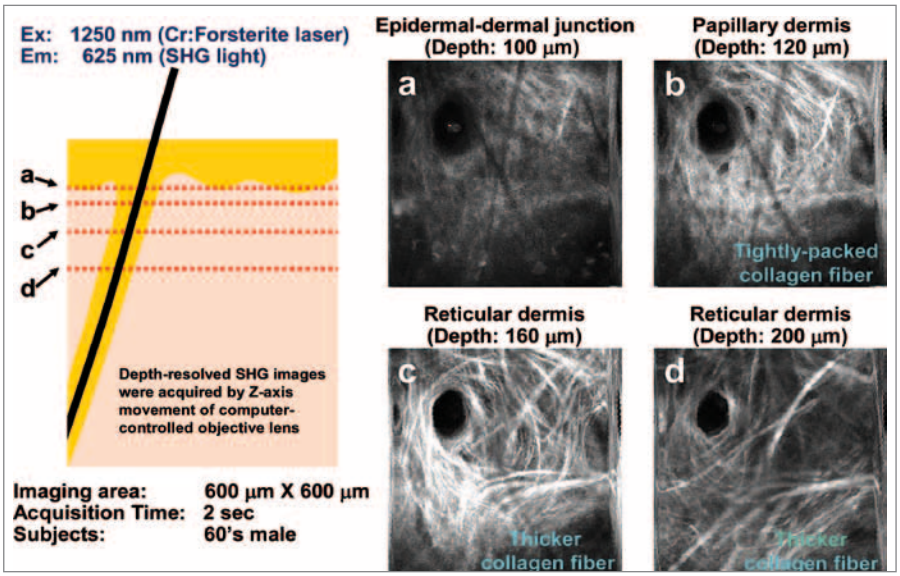

Fig. 5 Depth-resolved SHG images. Serial SHG images at the different depths were acquired from male subjects in their sixties: (a) Epidermal-dermal junction at the depth of 100 µm, (b) Papillary dermis at 120 µm, (c) Reticular dermis at 160 µm, (d) Reticular dermis at 200 µm

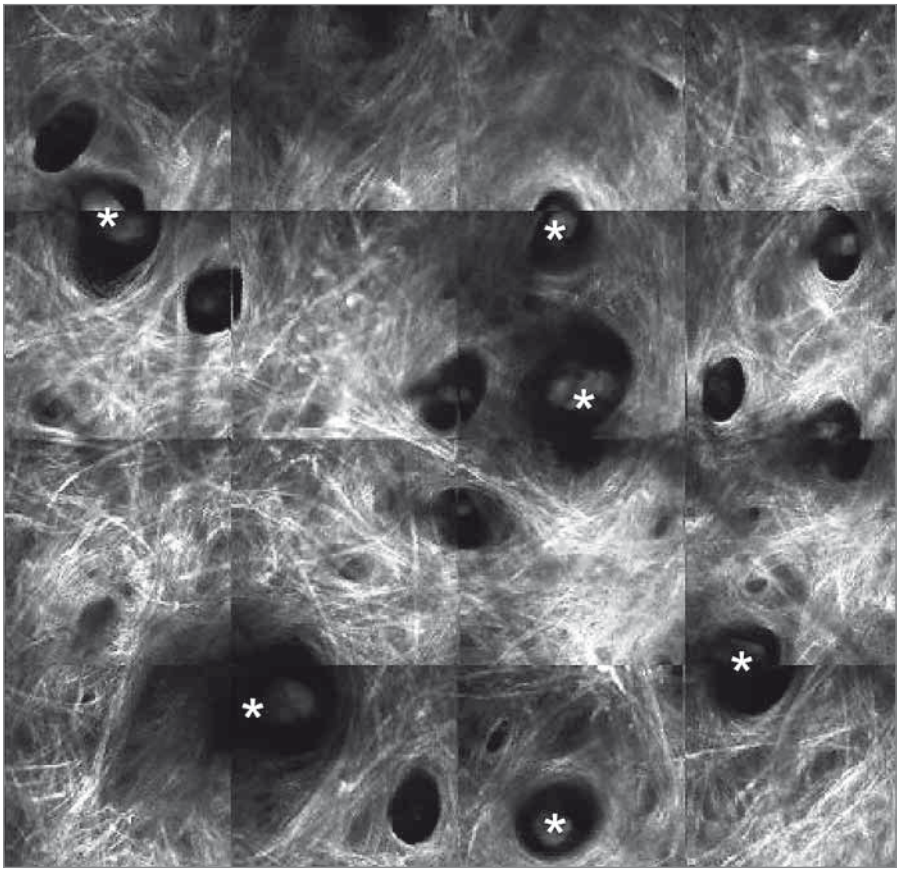

Fig. 6 Wide field of the collagen fiber image acquired by SHG microscopy. Sixteen consecutive SHG images of dermis at the depth of approximately 200 µm were obtained by computer-controlled 2D XY-stage movement (imaging area 2.4 X 2.4 mm, major hair follicles denoted by asterisks)

Age-related alteration of collagen fibers

Focusing on the dermal structural alteration as a sign of photodamage, cheeks of healthy female subjects of different age were evaluated using the SHG microscope. Photographs of individual cheek and representative SHG images are shown in **Fig. 7**. Comparison of the SHG images at the depth of 200 µm (reticular dermis) showed a trend in the degree of reflection to decrease with age. An SHG image of a female subject in her 20s showed a strong reflection over the whole region, apart from hair follicles, suggesting a high density of collagen fibers in the reticular dermis **(Fig. 7, a2)**. On the other hand, that of a female subject in her fifties showed a mesh-like texture pattern **(Fig. 7, d2)**. In this SHG image, individual collagen bundles were easily observed, suggesting a low density of collagen fibers due to loss of tiny collagen fibers. Additionally, the parameter of the collagen density (spectrum width; FWHM) calculated from Fouriour-transformed SHG images also supported a decreasing collagen density with age because the value of the spectrum width was relatively narrow in the dense distribution image pattern.

Moreover, to clarify how UV affects dermal collagen fibers in humans, we evaluated a female subject with a »high cumulative level of UV exposure to face (H-UV)«, and that with a »low cumulative level (L-UV)«. Magnified collagen images of a subject with a high cumulative level showed hardly observable collagen fibers except for some thicker fibers **(Fig. 8a)**, while an age-matched subject with a low cumulative level showed abundant fine collagen fibers **(Fig. 8b)**. These results indicate that SHG microscopy can be used in the evaluation of dermal photodamage due to cumulative UV exposure. Also, the visualized collagen fiber network is hardly observed in vertical-stained sections of skin specimens with conventional histological techniques. Therefore, en face SHG images will be useful for evaluating the collagen architecture in detail.

CONCLUSION

Noninvasive assessments of dermal structure in human skin were performed using a novel collagen-specific SHG microscopy technique. A wide field of en face collagen fiber images obtained by this newly developed SHG microscopy enabled us to evaluate photodamaged collagen fibers in human skin. In conclusion, the proposed

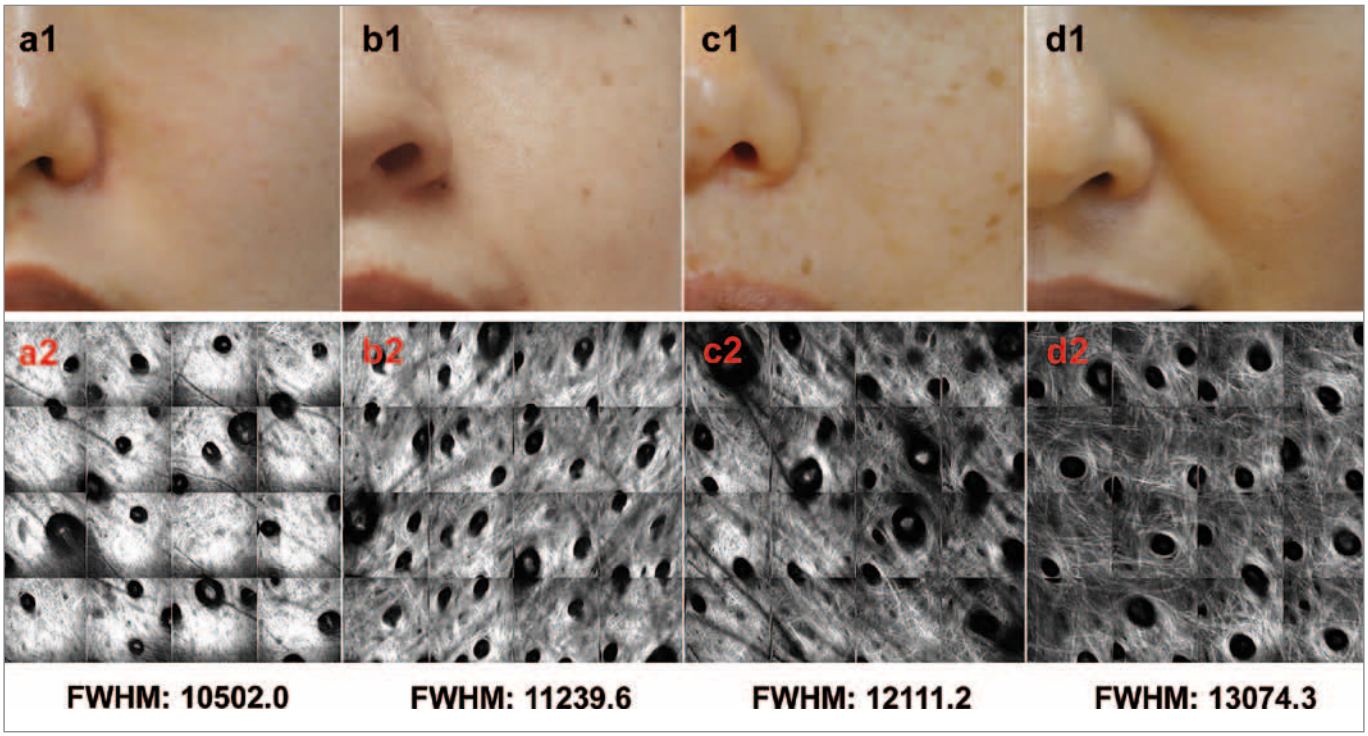

Fig. 7 Photographs and SHG images of female subjects of different age. Upper panels - photographs of individual cheek; lower panels - wide field of en face SHG images at the depth of approximately 200 µm. (a1 and a2) - subject in her 20s, (b1and b2) - subject in her 30s, (c1 and c2) - subject in her 40s, (d1 and d2) subject in her 50s. The area of each SHG image is 2.4 X 2.4 mm and the FWHM (degree) unit 1/m

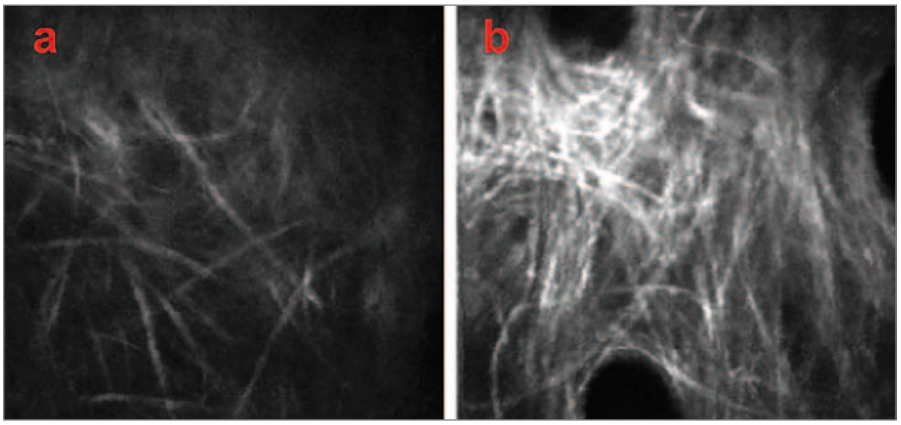

Fig. 8 Assessment of photodamage using SHG microscopy. (a) Female subject in her fifties with a high level of UV exposure (H-UV) who coaches outdoor sports; (b) age-matched female subject with a low level of UV exposure who is an office worker and had used sunscreen for many years. The depth of both SHG images is approximately 200 µm (reticular dermis) and the imaging area 600 x 600 µm

optical system promises to be a powerful tool to survey photoaging and monitor dermal alterations in UV-exposed skin. This cutting-edge technique focusing on extracellular matrix structure should also be useful for validation of the efficacy of suncare products against UV exposure.

References

[1] *Koenig, K.,* and *Riemann, I.,* High-resolution multiphoton tomography of human skin with subcellular spatial resolution and picosecond time resolution, J. Biomed Opt., 8 (2003) 432-439.

[2] *Tsai, T.H., Jee, S.H., Dong, C.Y.,* and *Lin, S.J.,* Multiphoton microscopy in dermatological imaging, J. Dermatol. Sci., 56 (2009) 1-8.

[3] *Yasui, T., Takahashi, Y., Ito, M., Fukushima, S.,* and *Araki, T., ex vivo* and *in vivo* second-harmonic-generation imaging of dermal collagen fiber in skin: comparison of imaging characteristics between mode-locked Cr:forsterite and Ti:sapphire lasers, Appl. Opt., 48 (2009) D88-95.

Corresponding author
Toyonobu Yamashita
Shiseido Research Center
Yokohama
Japan
toyonobu.yamashita@to.shiseido.co.jp