

Characterization of collagen orientation in human dermis by two-dimensional second-harmonic-generation polarimetry

Takeshi Yasui

Osaka University
Graduate School of Engineering Science
Toyonaka, Osaka 560-8531 Japan

Yoshiyuki Tohno

Nara Medical University
Department of 1st Anatomy
Kashihara, Nara 634-8521, Japan

Tsutomu Araki

Osaka University
Graduate School of Engineering Science
Toyonaka, Osaka 560-8531 Japan

Abstract. We have proposed an optical probe that can be used to characterize the orientation of collagen fibers in human dermis. A specific probing ability for collagen results from the use of second-harmonic-generation (SHG) light induced by collagen molecules in the tissue. Based on the concept of collagen SHG light, a reflection-type polarization measurement system (named SHG polarimetry) with a probe light spot of 15 μm in diameter has been constructed, and the human reticular dermis has been measured using this system. Resultant data exhibit that the reticular dermis possesses approximately uniaxial orientation of the collagen fibers. Furthermore, we demonstrated a nondestructive measurement of the collagen orientation in the papillary dermis across an epidermis layer. For distribution measurement of the collagen fiber orientation in the reticular dermis, we have extended the SHG polarimetry to one- (1-D) and two-dimensional (2-D) measurement. By the macroscopic 2-D SHG polarimetry, we have observed that the orientation angle and organization degree of collagen fibers vary widely depending on the discrete probing positions in the reticular dermis. Furthermore, microscopic 1-D SHG polarimetry indicated a swell of the orientation angle and a large variance of the organization degree in the collagen fibers in the microscopic region. These results imply that the reticular dermis possesses a tangled structure of collagen fibers, which is highly consistent with the result of the anatomical examination of the skin. The proposed method will be a powerful tool for monitoring the microscopic distribution of the collagen fiber orientation in the human dermis. © 2004 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.1644116]

Keywords: dermis; collagen; second-harmonic-generation light; femtosecond laser; orientation; polarization.

Paper JBO-014007 received Mar. 27, 2003; revised manuscript received Aug. 19, 2003 and Nov. 14, 2003; accepted for publication Nov. 17, 2003.

1 Introduction

Skin, the largest organ of the human body, is composed of a three-layer structure: epidermis, dermis, and subcutaneous tissue. Among these three layers, dermis plays a role in maintaining tension, elasticity, and water content physically in the skin. In the dermis layer consisting of reticular dermis and papillary dermis, a structural protein, collagen is abundant and contributes to the structural and functional properties of this layer. Since the structural change of collagen molecules and their aggregates (microfibrils, fibrils, fibers, and bundles) in the dermis provides important information about diseases afflicting tissues and organs such as skin cancer, collagen disease, or burns, there is a considerable need for an effective diagnostic technique by which the collagen structural changes in the dermis can be informed. Various methods that reveal the structure and orientation of collagen have been reported: electron microscopy,¹ x-ray diffraction,² microwave method,³ mechanical examination, biochemical, and histological analy-

sis. However, these methods are invasive and/or destructive because an anatomical tissue biopsy is required to complete the measurements. Although the biopsy-based methods are reliable for differentiating between normal and abnormal collagen structure, they inflict a heavy burden on the patient. Hence, there is a significant need for a noninvasive and nondestructive method as preliminary diagnosis to examine whether tissue biopsy is necessary or not.

Optical methods are attractive for such preliminary diagnosis, because they are simple, fast, and harmless to the patient. Since skin is a superficial tissue, an optical probe can be directly applied. However, the probing light signal is strongly scattered inside the tissue, and such the property makes the origin of detected light ambiguous. Surface reflection of the light is another obstacle. To overcome these problems, it is necessary to extract a weak but meaningful, less-scattered light from the mixture of the multiple-scattered light inside the sample and the surface-reflected light. A polarization gating technique is one possible method that eliminates the

Address all correspondence to Takeshi Yasui. Tel. +81-6-6850-6215; Fax: +81-6-6850-6212. E-mail: t-yasui@me.es.osaka-u.ac.jp

multiple-scattered light and/or surface-reflected light.^{4,5} This technique utilizes the property that the scattering events reduce the degree of polarization. However, it is difficult to characterize the collagen structure in the dermis from the resultant spectroscopic information in the extracted signal. Optical coherence tomography (OCT)⁶ is another possible method, and this method had been applied effectively to an optical probe of eye,⁶ skin,⁷ or digestive organ.⁸ In OCT, surface reflection and internal reflection can be distinguished easily, because resultant information for depth structure develops successively along the time axis. At the same time, unnecessary multiple-scattered light is effectively rejected by the coherence gate of light interference because the multiple scattering events reduce the coherency of light. Although OCT can probe microscopic structure in the collagen-containing tissues based on spatial variation in refractive index, it does not necessarily provide direct knowledge of the collagen fiber orientation in tissue. Polarization-sensitive OCT (PS-OCT)⁹ probes intrinsic birefringence and formation birefringence of the biological tissue. Hence, the PS-OCT gives direct information about the collagen fiber orientation and thus can be applied to the burn diagnosis of the skin.¹⁰ In addition to these linear optical probes, recent advances of ultrashort pulse lasers have opened the door to new optical probe methods based on the optical nonlinear effects in biological tissue: e.g., two-photon fluorescence,¹¹ second-harmonic-generation (SHG) light,¹² and coherent anti-Stokes Raman scattering (CARS) light.¹³ Among these linear and nonlinear optical probes, we believe that SHG light is favorable to the diagnosis of the collagen rich dermis. Because the SHG light efficiency and the concentration of the collagen molecule are much higher than those of other tissue components such as fat,¹⁴ the SHG light in the dermis is mainly due to collagen molecules. Such the collagen SHG light is generated only into the dermis layer because of no collagen content in the epidermis layer. In this situation, it is easy to reject surface reflection and multiple scattering of the incident laser light in the epidermis layer because the wavelength of the SHG light is half the incident laser wavelength. Hence, the collagen SHG light can give direct information about the collagen structure in the dermis without any gating technique. Moreover, the SHG light provides excellent three-dimensional resolution images and deep penetration power, and there are no photobleaching, phototoxicity, and additional staining with fluorochrome. The efficiency of SHG light changes with respect to the collagen orientation when the incident light is polarized, and hence, the polarization measurement of the SHG light is an effective tool that probes collagen orientation in the tissues.^{15,16} In this situation, the polarization measurement of the SHG light based on the reflection configuration (we named SHG polarimetry)¹⁷⁻¹⁹ is attractive when one achieves a noninvasive *in vivo* measurement of the collagen orientation in a thick living tissue. However, there are few reports dealing with determination of collagen orientation in actual human tissues based on that concept. We previously demonstrated the measurement of collagen orientation in the several human tissues by the SHG polarimetry, and presented remarkable differences among the tissues due to their characteristic collagen orientations.¹⁹

In this work, we present an optical probe method to study the collagen orientation in human reticular dermis and papillary dermis, based on SHG polarimetry. Using this method,

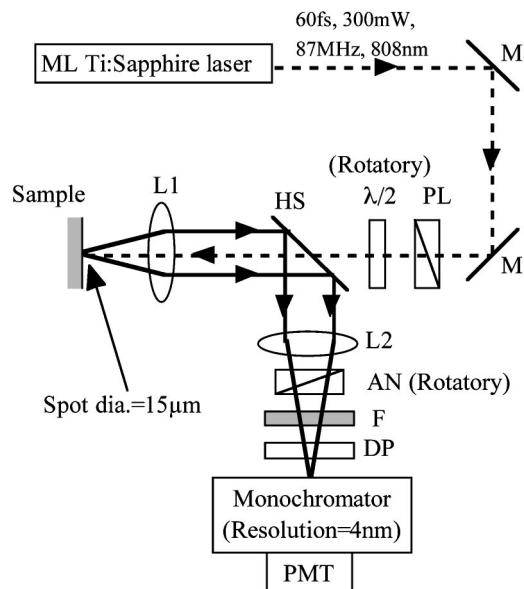


Fig. 1 Experimental setup. *M*: mirror, *PL*: polarizer, $\lambda/2$: half waveplate, *HS*: harmonic separator, *L1* and *L2*: lenses, *AN*: analyzer, *F*: blue-pass filter, *DP*: depolarizer, *PMT*: photomultiplier.

we also propose one-dimensional (1-D) and two-dimensional (2-D) SHG polarimetry to investigate the spatial distribution of collagen orientation in the reticular dermis.

2 Experimental Setup

Figure 1 shows the experimental setup for reflection-type SHG polarimetry. Instrumentation and basic performance of our SHG polarimetry are discussed elsewhere in detail.¹⁹ The laser source is a Kerr-lens-mode-locked Ti:sapphire laser (AVESTA TiF-kit-100, pulse width=60 fs, power=300 mW, repetition rate=87 MHz, center wavelength=808 nm). Ultrashort pulse light from the laser was focused onto the sample through a lens (*L1*, focal length=35 mm), resulting in a focused spot of 15 μm diameter in air. Although the SHG light induced in the sample mainly propagates in the same direction as the incident light, part of the SHG light is backscattered into the tissue, and then collected with *L1*. After elimination of the unnecessary laser light using a harmonic separator (*HS*) (reflected wavelength=400 nm) and a blue-pass filter (*F*) (pass wavelength=300–500 nm), the probing SHG light is spectrally resolved with a monochromator (resolution=4 nm) and detected with a photomultiplier (*PMT*). For polarization measurement of SHG light, a polarizer (*PL*), a half waveplate ($\lambda/2$), an analyzer (*AN*), and a depolarizer (*DP*) are inserted in the light path. The polarization direction of the laser light is rotated every 3 degrees by a mechanically rotatory $\lambda/2$ after adjusting to the linear polarization with the *PL*. Polarization of a detected SHG light is also rotated synchronously with the rotation of the laser light polarization to keep their polarization directions parallel to each other by a mechanically rotatory *AN*. The *DP* is attached to the rotatory *AN* to cancel the polarization dependence of the monochromator. The *HS* also causes slight anisotropy in transmittance and reflectivity for the polarization-rotating light. However, we did not consider such an effect in the

present work, because it is so small that the results of SHG polarimetry are not affected appreciably. For further strict qualification and quantification of the collagen orientation, such the anisotropy must be compensated. The described polarization-rotating procedure is equivalent to the rotation of the sample around the optical axis under fixed polarization optics. However, it was difficult to coincide the sample rotating axis and the optical axis within the probing spot. Hence, we adopted the polarization-rotating setup. Distribution of the SHG light intensity was measured with respect to the polarization angle. For imaging measurement of SHG light, a 2-D moving stage that scans the sample position laterally was adopted.

3 Samples

We prepared a human skin sample composed of epidermis and dermis (taken from cadaver's back), human Achilles tendon (from cadaver), and a collagen sponge for cell culture (Koken, CS-35, type I, from bovine Achilles tendon) as the sample specimens. Cadavers were treated by injecting a mixture of 36% ethanol, 13% glycerin, 6% phenol, and 6% formalin through the femoral artery. After an ordinary dissection by medical students in an anatomy class was finished, the skin was stripped off from the cadaver's back. The skin sample consisting of the epidermis and dermis was prepared by removing the subcutaneous tissues with a scalpel to expose the reticular dermis layer. The resulting skin sample, whose one side is the epidermis and another side is the exposed reticular dermis, was cut into a square sheet of 44 mm × 55 mm with 1 mm thick. The Achilles tendon, resected from the cadaver's leg, was sliced to 2 mm thick along the axial direction. After washing these tissue samples with distilled water, they were dried in air. These three kinds of sample were put between two glass slides to flatten the sample surface.

The Achilles tendon and collagen sponge, which possess uniaxial and completely random orientations in collagen fiber, respectively, were used as the control samples.

4 Results

4.1 Effects of Sample Preparation Procedures on SHG Polarimetry

Before determination of the collagen orientation in the human dermis, we have to investigate the effect of sample preparation procedures (formalin fixing and air drying) on SHG polarimetry, because such formalin-fixed human tissue samples have not been used generally in SHG methods.

We first evaluated effect of formalin fixing on the SHG polarimetry by preserving a native dermis tissue in a chip section of a raw chicken wing in the formalin-based fixation medium (36% ethanol, 13% glycerin, 6% phenol, and 6% formalin). Intensity distribution of the detected SHG light was measured as a function of polarization angle, and recorded as "SHG radar graph" shown in Fig. 2, where 0 (or 180) and 90 (or 270) degrees mean horizontal and vertical polarizations, respectively. The polarization angle of the major axis and the profile of the SHG radar graph reveal the direction of the absolute orientation and the organization degree of collagen fibers in the area of the light spot, respectively. Figures 2(a) and 2(b) show SHG polarimetry before and 5 h after such

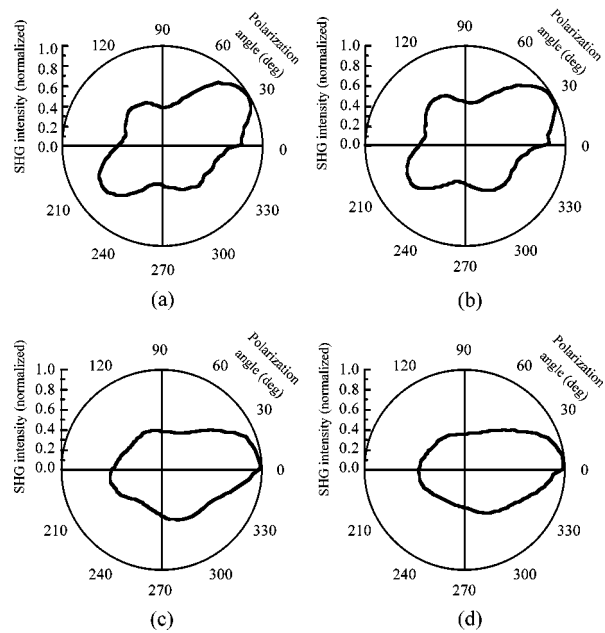


Fig. 2 Effects of sample preparation procedures on SHG polarimetry: (a) and (b) are SHG radar graphs before and 5 h after formalin fixation, (c) and (d) are those before and 13 h after air drying. Sample is a native dermis tissue in a chip section of a raw chicken wing.

fixation, respectively. As a result, the fixation process indicates almost unchanged in SHG polarimetry while the SHG intensity was slightly increased (not shown). Since formalin fixing induces crosslinking of collagen in tissues, this result may imply that the crosslinking does not affect collagen orientation essentially but contributes efficiency of SHG light.

We next investigated the effect of air drying on SHG polarimetry by leaving the earlier formalin-fixed chicken dermis sample in air for a long time. The SHG polarimetry before and 13 h after the air drying were shown in Figs. 2(c) and 2(d), respectively. One can confirm that SHG polarimetry was almost unchanged during such drying process. However, the SHG intensity was decreased to about a fourth (not shown). We think that the decrease of the SHG intensity results in change of a linear optical property, i.e., scattering efficiency, rather than that of efficiency of SHG radiation in the tissues.

From these two preliminary results, we conclude that the sample preparation procedures used in this paper do not affect the SHG polarimetry even if the SHG light intensity is influenced by them.

4.2 Comparison of Collagen Orientation Among Samples

Results of SHG polarimetry for the samples are shown in Fig. 3. Arrows in each polarimetry indicate the direction of the collagen orientation expected from the SHG radar graph. The human Achilles tendon indicates a typical "figure-eight" profile whose major axis is parallel to the collagen orientation as shown in Fig. 3(a). In general, collagen SHG light is strongly outputted with the same polarization as the incident light, when the polarization direction of the incident light was parallel to the longitudinal direction of the collagen fiber. However, the SHG light almost disappears if the laser polarization

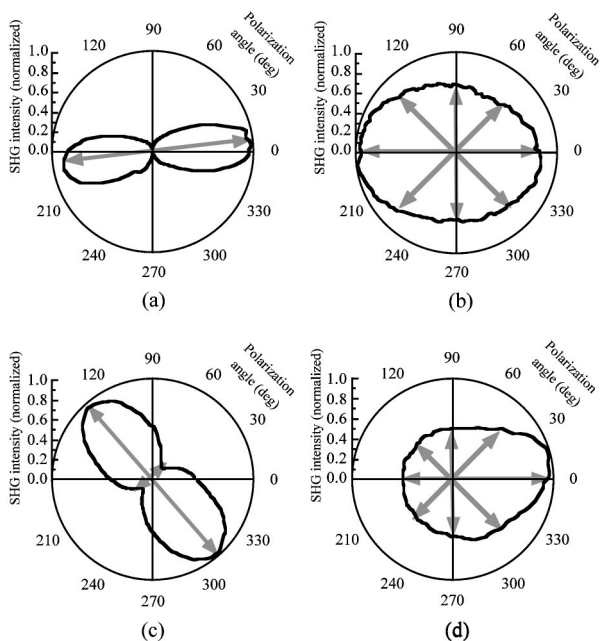


Fig. 3 SHG polarimetry profiles for tissue samples: (a) human Achilles tendon, (b) collagen sponge, (c) human reticular dermis, and (d) human papillary dermis. Arrows in each graph indicate expected direction of collagen orientation.

is perpendicular to the collagen orientation, because the second optical nonlinear property along this direction is considerably low. Hence, such a figure-eight profile is characteristic of a uniaxial fiber orientation.¹⁹ The oval profile of the collagen sponge implies completely random orientation of the collagen fibers [Fig. 3(b)], because this results in that SHG intensity is approximately equal for all polarization angles. We note here that small asymmetry in the SHG radar graph is due to imperfect optical alignment in the experimental setup because the SHG polarimetry is very sensitive to small alignment errors (for example, incomplete normal incidence of the SHG light to the analyzer). For the dermis, we studied the collagen orientation by two different approaches. In the first approach, the laser light was incident from a subcutis side of the skin sheet sample, i.e., bare reticular dermis, and the SHG light from it was measured. Although the resulting profile of the reticular dermis is similar to that of the Achilles tendon, its figure-eight profile is lacking a node [Fig. 3(c)]. This result indicates that the orientation of the collagen fiber in the reticular dermis is not completely uniaxial such as the Achilles tendon, but nearly uniaxial. In general, anatomical findings of the reticular dermis indicate that it possesses tangled structure of collagen fibers in every direction. We think that such a tangled degree of the collagen fiber within the probing area of 15 μm makes the imperfect figure-eight profile in the SHG polarimetry. The second approach is more challenging, that is, probing of the dermis collagen orientation across the epidermis layer. In this approach, the laser light is incident from the epidermis side. When the laser light was focused on the surface of the epidermis, the collagen SHG light was not observed. However, if the probing spot was moved to a deeper region and reached the dermis layer, the SHG light signal appeared. Since keratin in the epidermis does not radiate the

SHG light,²⁰ this SHG light was originated from the collagen molecule in the dermis interacted with the laser light passing through the epidermis. The resulting SHG radar graph in Fig. 3(d) indicates a similar shape to that of the collagen sponge in Fig. 3(b) but with eccentric characteristics. Also, one can confirm a distinct difference from that of the reticular dermis in Fig. 3(c). Such a difference might be due to the difference of the collagen orientation between reticular and papillary dermis, because we probed a boundary between the epidermis and the dermis, that is, a papillary dermis rich layer. Although it is also necessary to consider the effects of multiple scattering into the epidermis layer, i.e., decrease of spatial resolution and depolarization of the laser light, the present result implies a possibility of nondestructive, *in vivo* monitoring of the dermis collagen in clinical medicine.

Here, to quantify the collagen orientation in the tissue, let us lead two parameters, θ_{maj} and PA, obtained from the SHG radar graph. The θ_{maj} value is defined as a polarization angle of the major axis in the SHG radar graph. For organization degree of the collagen orientation, we define a polarization anisotropy of the SHG light (PA) as,

$$\text{PA} = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp}), \quad (1)$$

where I_{\parallel} and I_{\perp} are the SHG intensity at the θ_{maj} and that perpendicular to the θ_{maj} , respectively. Collagen orientation is unidirectional for PA=1, while it is isotropic directional for PA=0. The θ_{maj} values for the Achilles tendon and reticular dermis are 6.5 and 131 degrees, respectively (see Fig. 3). Since the collagen sponge and papillary dermis did not indicate a clear major axis, the θ_{maj} values for them were omitted. On the other hand, the PA values were 0.89 for the Achilles tendon, 0.13 for collagen sponge, 0.74 for reticular dermis, and 0.15 for papillary dermis. One can see a significant difference of the PA value among the four specimens. Such a difference is due to the collagen orientation. We can quantitatively expect the orientation conditions of collagen fibers from the sample dependent θ_{maj} and PA values.

4.3 Macroscopic and Microscopic Lateral Distribution of Collagen Orientation

Dermis exists as a sheet of collagen-rich tissue, and acts as a cover and protector of the inner tissues and organs. Therefore, the lateral distribution of the collagen orientation is interesting when considering the structural and functional properties of the dermis. From this point of view, we extended SHG polarimetry to 1-D and 2-D measurement to further investigate the structural orientation of collagen fibers in the dermis. Here, we probed the exposed reticular dermis. The present SHG polarimetry requires a measurement time of 1 min/spot because of the time required for the mechanical rotation of polarization optics, resulting in considerable time consumption for precise two-dimensional measurement. The acquisition time can be shortened dramatically by controlling the polarization with electro-optical devices instead of mechanical ones. In spite of the limitation of the present system, we will demonstrate here the primitive measurement of 20 spots by SHG polarimetry on the reticular dermis. Mapping of SHG radar graphs is shown in Fig. 4, where the spot measurement was made every 10 mm on the sample sheet of 40 mm \times 55 mm. Although each profile showed orientation profiles

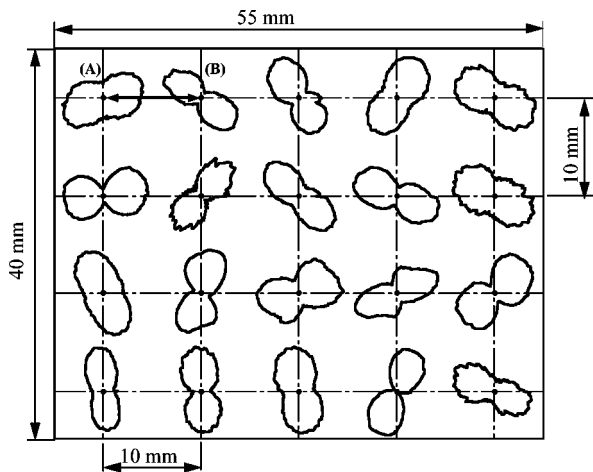


Fig. 4 Macroscopic 2-D SHG polarimetry in reticular dermis. Measurement was done in spot areas at 10 mm intervals on dermis sheet of 55 mm×40 mm.

similar to Fig. 3(c), we found a slight difference in the degree of organization, depending on the probing position. Furthermore, the angle of the major axis was not ordered. Such characteristic collagen distribution in the reticular dermis was completely different from those in the Achilles tendon and collagen sponge that indicate uniform distribution of collagen

orientation throughout (not shown). We approximate that the reticular dermis has nearly uniaxial collagen fiber orientation in a microscopic region of the scale of the probing spot (15 μm diameter), while such oriented portions are randomly distributed throughout the macroscopic region. This result partially indicates an entangled structure of the collagen fiber in the reticular dermis.

Figure 4 gives us the intuitive findings of macroscopic and discrete lateral distribution of the collagen fiber orientation in the reticular dermis. On the other hand, it is also interesting to probe the continuous distribution of the collagen orientation in the microscopic region. Hence, we next interpolate a microscopic lateral distribution of the collagen orientation between the two discrete probing locations [(A) and (B)] in Fig. 4 by microscopic 1-D SHG polarimetry. The probing light spot was one-dimensionally moved stepwise in 100 μm intervals from (A) to (B) by operating the scanning stage. Microscopic distribution of the collagen orientation was evaluated using the θ_{maj} and PA values as shown in Figs. 5(a) and 5(b), respectively. These values in the reticular dermis are compared with those in the Achilles tendon. Furthermore, the position region from 0 to 1-mm in Figs. 5(a) and 5(b) is expanded by 10 μm step scanning in Figs. 5(c) and 5(d). Statistics of these values in position ranges of 0–10 and 0–1 mm are summarized in Table 1. The θ_{maj} value in the Achilles tendon is almost constant in both ranges, indicating well-ordered axial orientation in the collagen fiber in the micro-

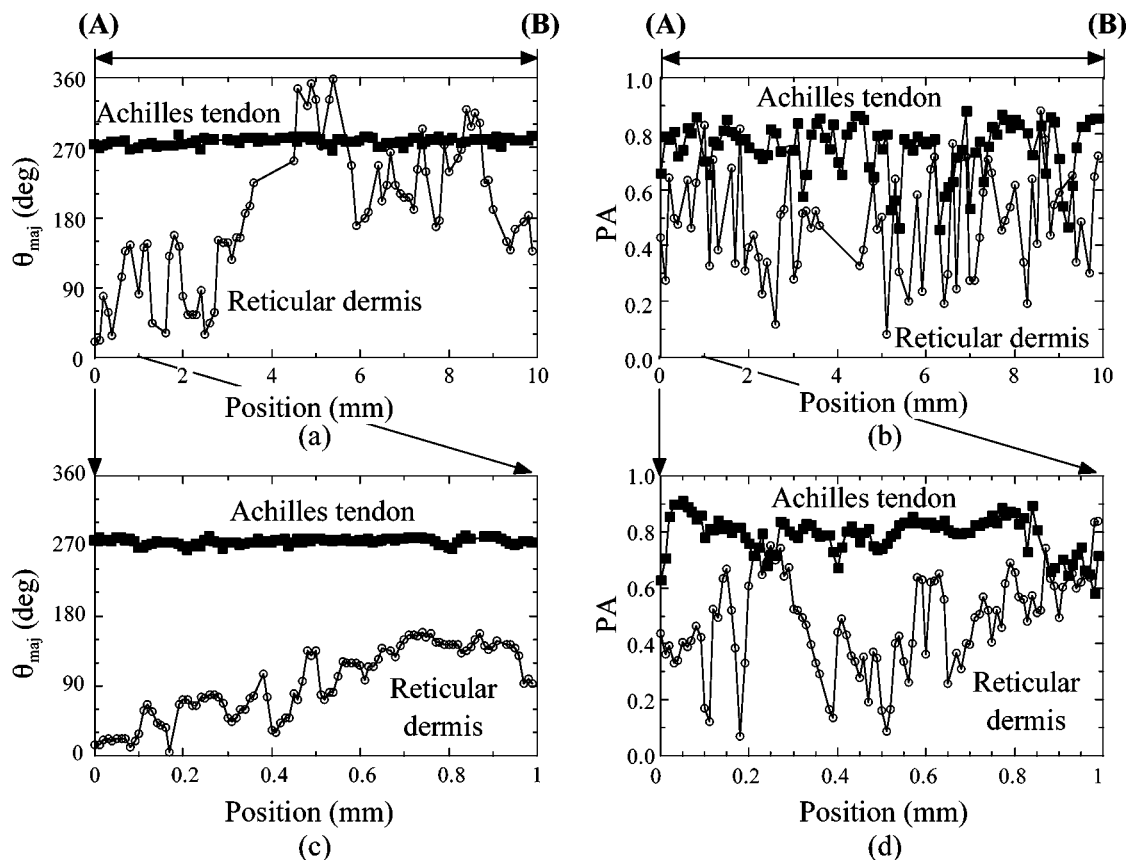


Fig. 5 Microscopic lateral distribution of collagen orientation in reticular dermis: (a) θ_{maj} value and (b) PA value within region of 0–10 mm, and (c) θ_{maj} value and (d) PA value within region of 0–1 mm. The θ_{maj} and PA values inform direction angle and organization degree of collagen orientation, respectively. Results of the reticular dermis were compared with those of the Achilles tendon.

Table 1 Comparison of θ_{maj} and PA values in reticular dermis and Achilles tendon.

Parameter	θ_{maj} (deg, mean \pm SD)		PA (mean \pm SD)	
	0–1 mm	0–10 mm	0–1 mm	0–10 mm
Reticular dermis	92.1 \pm 46.0	180.5 \pm 90.2	0.48 \pm 0.17	0.49 \pm 0.18
Achilles tendon	275.3 \pm 3.8	277.4 \pm 4.3	0.79 \pm 0.07	0.75 \pm 0.10

scopic region. The reticular dermis produced an interesting swell and drift of the θ_{maj} value in addition to its variation in each range. For the PA value, we observed significant differences between the two samples. Mechanical property of the Achilles tendon is supported by the spatial distribution of a high PA value. We confirmed a large variance of deviations around PA=0.5 in the reticular dermis. From such a characteristic swell of the θ_{maj} value and a large variance of the PA value, we can imagine that the collagen fibers in the reticular dermis become entangled successively within these spatial scales, which is in good agreement with the anatomical findings of the dermis.

5 Conclusion

We have proposed 1-D and 2-D SHG polarimetry to evaluate spatial distribution of the collagen orientation in the human dermis. On the basis of the imperfect figure-eight profile obtained in SHG polarimetry, collagen fibers in the reticular dermis were characterized as having approximately uniaxial orientation. We also demonstrated the probing of the collagen orientation in the papillary dermis across the epidermis, which provides a potential of *in vivo* monitoring of the dermis collagen in clinical medicine.

Distribution measurement of the collagen orientation using macroscopic 2-D and microscopic 1-D SHG polarimetry indicated that the reticular dermis exhibits almost a uniaxial collagen orientation within a microscopic region and such the collagen orientation becomes gradually disordered with the increase of the probing area due to the mixing of the various orientations. From these results, we conclude that the proposed measurement system is a powerful tool for the monitoring of the structural orientation of the collagen fibers in the human dermis.

Acknowledgments

Portions of this work were supported by a Grant-in-Aid for Scientific Research Nos. 15650094 and 15300161, from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors would like to thank K. Shimabayashi and H. Kawaguchi for their technical help in experiments.

References

1. B. Eyden and M. Tzaphlidou, "Structural variations of collagen in normal and pathological tissues: role of electron microscopy," *Micron* **32**, 287–300 (2001).
2. V. J. James, L. Delbridge, S. V. McLennan, and D. K. Yue, "Use of

- x-ray diffraction in study of human diabetic and aging collagen," *Diabetes* **40**, 391–394 (1991).
3. S. Osaki, "Distribution map of collagen fiber orientation in a whole calf leather," *Anat. Rec.* **254**, 147–152 (1999).
4. J. M. Schmitt, A. H. Gandjbakhche, and R. F. Bonner, "Use of polarized light to discriminate short-path photons in a multiply scattering medium," *Appl. Opt.* **31**, 6535–6546 (1992).
5. S. P. Morgan and M. E. Ridgway, "Polarization properties of light backscattered from a two layer scattering medium," *Opt. Express* **7**, 395–402 (2000).
6. D. Huang, E. A. Swanson, C. P. Lin, J. S. Schuman, W. G. Stinson, W. Chang, M. R. Hee, T. Flotte, K. Gregory, C. A. Puliafito, and J. G. Fujimoto, "Optical coherence tomography," *Science* **254**, 1178–1181 (1991).
7. J. M. Schmitt, M. Yadlowsky, and R. F. Bonner, "Subsurface imaging of living skin with optical coherence microscopy," *Dermatology (Basel, Switz.)* **191**, 93 (1995).
8. A. M. Rollins, R. Ung-Arunyawee, A. Chak, R. C. K. Wong, K. Kobayashi, M. V. Sivak, and J. A. Izatt, "Real-time *in vivo* imaging of human gastrointestinal ultrastructure by use of endoscopic optical coherence tomography with a novel efficient interferometer design," *Opt. Lett.* **24**, 1358–1360 (1999).
9. J. F. de Boer, T. E. Milner, M. J. C. van Gemert, and J. S. Nelson, "Two-dimensional birefringence imaging in biological tissue using polarization sensitive optical coherence tomography," *Opt. Lett.* **22**, 934–936 (1997).
10. J. F. de Boer, S. M. Srinivas, A. Malekafzali, Z. Chen, and J. S. Nelson, "Imaging thermally damaged tissue by polarization sensitive optical coherence tomography," *Opt. Express* **6**, 212–218 (1997).
11. W. Denk, J. H. Strickler, and W. W. Webb, "Two-photon laser scanning fluorescence microscopy," *Science* **248**, 73–76 (1990).
12. S. Fine and W. P. Hansen, "Optical second harmonic generation in biological tissues," *Appl. Opt.* **10**, 2350–2353 (1971).
13. A. Zumbusch, G. R. Holtom, and X. S. Xie, "Three-dimensional vibrational imaging by coherent anti-Stokes Raman scattering," *Phys. Rev. Lett.* **82**, 4142–4145 (1999).
14. B. M. Kim, J. Eichler, and L. B. Da Silva, "Frequency doubling of ultrashort laser pulses in biological tissues," *Appl. Opt.* **38**, 7145–7150 (1999).
15. S. Roth and I. Freund, "Second harmonic generation in collagen," *J. Chem. Phys.* **70**, 1637–1643 (1979).
16. P. Stroller, K. M. Reiser, P. M. Celliers, and A. M. Rubenchik, "Polarization-modulated second harmonic generation in collagen," *Biophys. J.* **82**, 3330–3342 (2002).
17. P. Stroller, B.-M. Kim, A. M. Rubenchik, K. M. Reiser, and L. B. Da Silva, "Polarization-dependent optical second-harmonic imaging of a rat-tail tendon," *J. Biomed. Opt.* **7**, 205–214 (2002).
18. T. Yasui, K. Shimabayashi, H. Kawaguchi, Y. Tohno, T. Iwata and T. Araki, "Polarimetry of second-harmonic-generation light sensitive to orientation of tissue collagen," *Abst. International Conference on Optics Within Life Science VII*, Switzerland, 2002, p. 19.
19. T. Yasui, Y. Tohno, and T. Araki, "Determination of collagen fiber orientation in human tissue by polarization measurement of molecular second-harmonic-generation light," (unpublished).
20. P. J. Campagnola, A. C. Millard, M. Terasaki, P. E. Hoppe, C. J. Malone, and W. A. Mohler, "Three-dimensional high-resolution second-harmonic generation imaging of endogenous structural proteins in biological tissues," *Biophys. J.* **82**, 493–508 (2002).