Determination of collagen fiber orientation in human tissue by use of polarization measurement of molecular second-harmonic-generation light

Takeshi Yasui, Yoshiyuki Tohno, and Tsutomu Araki

Based on the reflection-type polarization measurement of second-harmonic-generation (SHG) light induced by collagen molecules, we are able to determine the collagen fiber orientation in human tissues taken from a cadaver. The resulting SHG radar graph shows the direction of the absolute orientation and the degree of organization of collagen fibers. To evaluate the probing sensitivity to the collagen orientation, we compared the proposed method with other polarimetric methods. Use of the proposed method revealed characteristic orientation differences among collagen fibers and demonstrated significant inhomogeneity with respect to the distribution of collagen orientation in human dentin. The proposed method provides a powerful research and diagnostic tool for examining the collagen orientation in human tissues. © 2004 Optical Society of America

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1. Introduction

Collagen is an important structural protein in the human body, and its molecules form structural aggregates successively (i.e., microfibrils, fibrils, fibers, and bundles of different sizes) in biological tissues and organs. Because collagen molecules and their aggregates play a role in maintaining the mechanical property of tissues in a way similar to that of reinforcing bars in a concrete building, the orientation of the collagen molecule aggregates is closely related to the structural and functional properties of the tissues and organs. For this reason, there is considerable need in the areas of biological research and clinical medicine for a reasonable method that can reveal the collagen orientation *in situ*. For example, in skin grafting for severe burns, the collagen orientation of the grafted dermis should be consistent with that of the dermis surrounding the burn. Even a small inconsistency in collagen orientation causes a difference in the mechanical property at the boundary of the two dermises, resulting in ugly wrinkles in the skin. However, in the case of conventional skin grafting surgery, an accurate determination of the collagen orientation depends on the doctor's experience and skill.

Several studies describe the methods for observing the collagen orientation: electron microscopy,[1] x-ray diffraction,[2] microwave method,[3] mechanical examination, and biochemical and histological analyses. Because a tissue biopsy is required for measurement purposes, these methods are invasive and/or destructive and place a heavy burden on the patient. Optical probe methods offer the possibility of noninvasive and nondestructive diagnosis of the structural changes in biological tissues. For example, optical coherence tomography (OCT) has been effectively applied to the optical biopsy of eyes,[4] skin,[5] and digestive organs.[6] On the other hand, recent advances in ultrashort pulse lasers have opened the door to new optical probe methods based on the optical nonlinear interaction between the incident light field and the analytical substance: two-photon fluorescence,[7] second-harmonic-generation (SHG) light,[8] and coherent anti-Stokes Raman scattering light.[9] Among these probes, SHG light is preferred for the diagnosis of collagen-rich tissues,[10–14] because it readily provides direct information about the collagen structure in the tissues without photobleaching, phototoxicity, and addi-

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tional staining with fluorochrome. The efficiency of SHG light is sensitive to the collagen orientation when the incident light is polarized,[10] and hence the polarization measurement of SHG light is effective in probing the collagen orientation in the tissues.[10, 13, 14]

As a noninvasive *in vivo* measurement of a thick living tissue, the polarization measurement of SHG light based on the reflection configuration (called SHG polarimetry) is attractive.[15–18] Although this configuration has been applied to the determination of the collagen orientation in animal tissues,[17, 18] few reports deal with human tissues. When one considers the use of SHG polarimetry as the optical probe combined with a biopsy technique for human tissues, it is better to evaluate the proposed system's sensitivity to the collagen orientation in human tissue rather than in animal tissue. However, living human tissue is not readily available because of the ethics guidelines governing its use. In a previous study[19] we examined whether there is an appreciable difference between the SHG polarimetry of formalin-fixed tissue and fresh tissue and confirmed that the SHG polarimetry profile of the fixed sample was consistent with that of the unfixed sample. This result implies the applicability of the SHG polarimetry to living human tissues.

In the present paper, as a first step for future medical applications, we demonstrate the measurement of the collagen orientation in human cadaver tissues by use of SHG polarimetry. The probing sensitivity of the SHG polarimetry to the collagen orientation is evaluated by comparison with that of other polarimetric methods. Furthermore, we discuss the distribution of the collagen orientation in human dentin in the context of tooth aging.

2. Experimental Setup

Figure 1 shows the experimental setup for the SHG polarimetry. An infrared pulse light with ultrahigh peak power and ultrashort pulse width is required to generate the collagen-induced SHG light efficiently without causing thermal damage to the tissue. We used an 87-MHz Kerr-lens mode-locked Ti:sapphire laser (Avesta Project, Ltd., TiF-Kit-100) pumped by a 5-W frequency-doubled Nd:YVO_4 laser (Showa Optronics, JUNO5000) running at 532 nm. The laser pulse has a duration of 60 fs and an average power of 300 mW at 808 nm. After passing through an optical chopper (OCP, chopping frequency of 3 kHz), ultrashort pulse light from the laser is focused onto the sample through a lens (L1, focal length of 35 mm), resulting in an analytical spot of 15-μm diameter and 450-μm depth in air. Although SHG light that is induced in the sample propagates mainly in the same direction as the incident light, a portion of SHG light is backscattered into the tissue and then collected via L1. After unnecessary laser light is eliminated with a harmonic separator (HS, reflected wavelength of 400 nm) and a blue-pass filter (F, pass wavelength of 300–500 nm), the analytical SHG light is spectrally

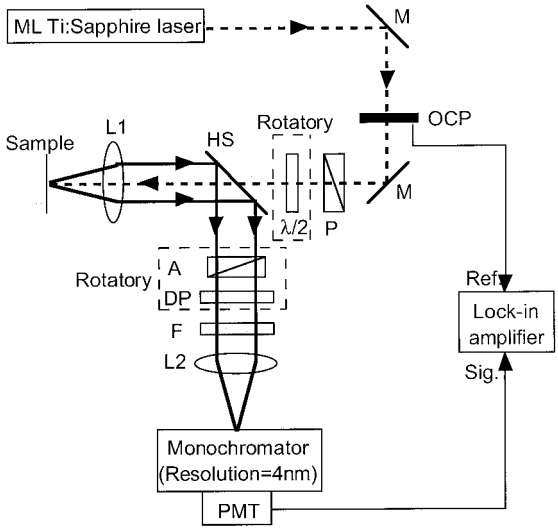

Fig. 1. Experimental setup: ML, mode-locked; M, mirror; L2, lens; Ref., reference input; Sig., signal input. See text for other definitions. For SHG polarimetry, polarizations of the incident laser light and the detected SHG light are simultaneously rotated while they are kept parallel to each other.

resolved with a monochromator (resolution of 4 nm) and detected with a photomultiplier (PMT) and a lock-in amplifier.

For the polarization measurement of SHG light, the polarization direction of the laser light was rotated every 3 deg with a mechanically rotatory half-wave plate (λ/2) after adjustment to the linear polarization with a polarizer (P). The polarization of a detected SHG light was also rotated synchronously with the rotation of the laser light polarization by means of a mechanical rotatory analyzer (A) in order to maintain the polarization directions parallel to each other. A depolarizer (DP) was attached to the rotatory analyzer to cancel the polarization dependence of the monochromator. The distribution of the SHG light intensity was measured with respect to the polarization angle. Although a rotation procedure such as this was equivalent to the rotation of the sample around the optical axis under fixed polarization optics, it was difficult to coincide the sample rotating axis with the optical axis within the analytical spot. Hence we selected a polarization-rotating setup. In this setup we also had to evaluate the influence of the anisotropy in the HS that is induced by transmission and reflection of the polarization-rotating light. The polarization-rotating 800-nm light is influenced by anisotropic difference of ±2.3% in transmittance, whereas the polarization-rotating 400-nm light is subjected to anisotropy of ±2.8% in reflectance. However, we did not need to compensate for the anisotropic difference in the practical polarimetry because the small difference did not so seriously affect the results of SHG polarimetry. To achieve a more precise characterization of the collagen orientation with the proposed method, one must compensate the anisotropy by using a nonoriented SHG material (i.e., a powder of SHG crystal or optically

active material) whose generation efficiency of SHG light is unchanged by the polarization-rotating light.

3. Samples

We prepared four kinds of collagen-rich human tissue samples: Achilles tendon (from a cadaver), dermis (from a cadaver), dentin (extracted from a patient by a dentist), and talus (from a cadaver). Each tissue has one of the following characteristic collagen fiber orientations: (1) unaxial orientation, (2) entangled orientation, (3) biaxial orientation due to the mesh structure, or (4) parallel-to-trabecule collagen distribution. Cadavers were given an injection of a mixture of 36% ethanol, 13% glycerin, 6% phenol, and 6% formalin through the femoral artery. After a typical anatomy class dissection had been carried out by medical students, the tendon and skin were resected. Once these tissue samples were washed with distilled water, they were dried in air. We have confirmed that these sample preparation procedures (formalin fixation and air-drying) do not affect the results of SHG polarimetry.[19] Two kinds of tendon specimens, each 2-mm thick, were prepared: one was sliced along the axial direction and the other across the section. The skin was stripped off the cadaver's back, and the subcutaneous tissue was removed with a scalpel to expose the reticular dermis layer. The resulting skin sheet sample, consisting of the epidermis and the exposed reticular dermis, was cut into a 44 mm $\times$ 55 mm square sheet of 1-mm thickness. The dentin and talus were sliced to a 1-mm thickness along the tooth axis and to a 2-mm thickness across the section by use of a tooth cutter and a bone cutter, respectively. A raw chicken skin was prepared as a native, wet biological tissue. We also prepared a collagen sponge for cell culture (Koken, CS-35, type I, from bovine Achilles tendon). The Achilles tendon samples, which possess well-defined uniaxial collagen orientations, were used as the control.

4. Second-Harmonic-Generation Polarimetry

A. Relationship between Incident Laser Polarization and Collagen Orientation

The collagen-induced SHG light is produced by the specific structure of collagen, that is, by the absence of a center of inversion symmetry on the scale of light wavelength. The SHG phase-matching condition is not achieved in the biological tissue owing to the small birefringence of the collagen fiber.[20] Alternatively, the collagen-induced SHG light is in the nonphase-matching condition, in which SHG light from each collagen molecule is constructively interfered and hence coherently enhanced by multiple scattering within a coherent length in the tissue.[11]

The relation between the collagen orientation and the laser polarization with respect to SHG light is summarized in Fig. 2. When an incident light is normal to the cross section of the collagen fiber, the collagen SHG light is not observed at any polarization angle. In an incidence configuration such as this, the cylindrical symmetry (C_∞ symmetry) along the longitudinal axis in collagen molecules makes any laser polarization centrosymmetric with respect to the collagen orientation, resulting in the prohibition of SHG light. However, other configurations induce SHG light because the relation between the collagen orientation and the laser polarization is noncentrosymmetric. In particular, when the laser polarization is parallel to the collagen orientation, a strong SHG light is observed. The polarization direction of the resultant SHG light is consistent with that of the incident laser light. On the other hand, SHG light is very weak when the laser polarization is perpendicular to the collagen orientation. These polarization dependences reflect the nonzero elements of the second-order susceptibility tensor ($\chi^{(2)}$) in collagen molecules, the analytical model of which is discussed elsewhere.[10, 17]

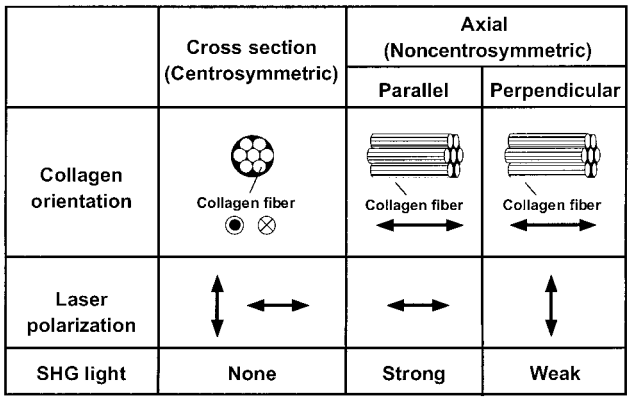

Fig. 2. Relationship between collagen orientation and laser polarization with respect to the generation of SHG light.

To confirm these relations, we measured the collagen SHG light on the human Achilles tendon, which possesses a well-defined uniaxial orientation in the collagen fiber. As expected, the resultant SHG light output was strong when the polarization direction of the incident light was parallel to the longitudinal fiber direction and considerably weaker when the laser polarization and the collagen orientation were perpendicular to each other (ratio of SHG intensity between the former and the latter configurations of 50:1). This result indicates that the SHG polarimetry is a probe specific to the collagen orientation.

B. Comparison with Other Polarimetric Methods

To evaluate SHG polarimetry, we compared the probing sensitivity to the collagen orientation with other polarimetric methods: (a) reflected fundamental light, (b) transmitted fundamental light, and (c) two-photon excitation autofluorescence (TPEAF). Experiments using these methods employ the same experimental setup as the SHG polarimetry. Vertically and horizontally directed human Achilles tendons were used as the standard samples. Figure 3 shows the resultant radar graphs of the four methods with respect to the distribution of the detected light intensity as a function of polarization angle, where 0 (or 180) and 90 (or 270) deg indicate the horizontal

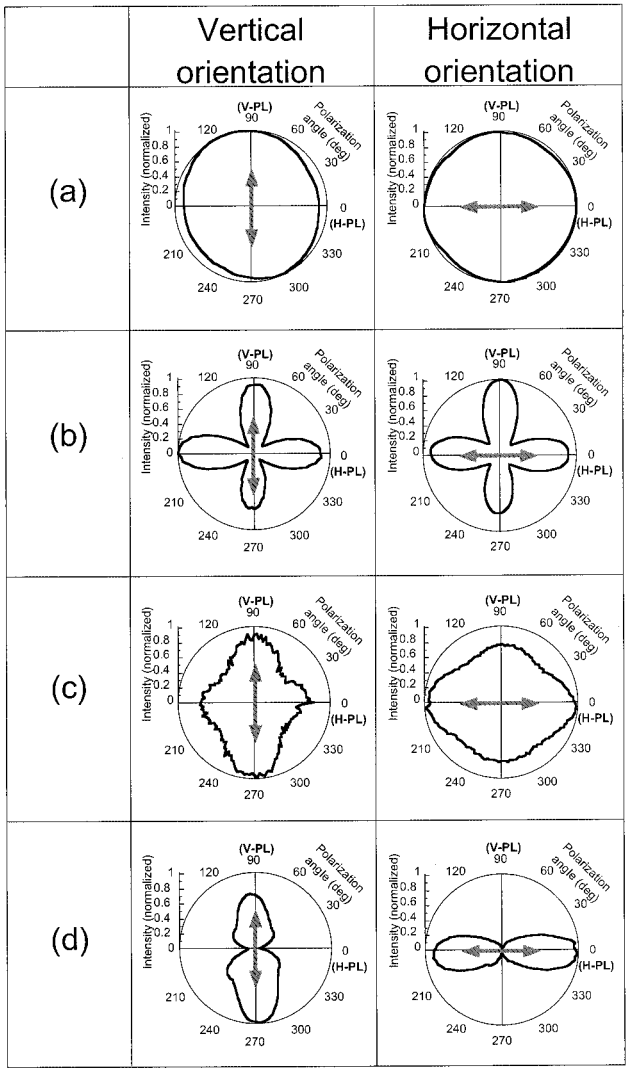

Fig. 3. Comparison of the SHG polarimetry with other polarimetric methods: (a) reflected fundamental light, (b) transmitted fundamental light, (c) TPEAF, and (d) SHG. The human Achilles tendon is used as a sample with an uniaxial collagen orientation. Gray arrows in each graph indicate the actual direction of the collagen orientation.

and vertical polarizations, respectively. The gray arrows in each graph indicate the actual direction of the collagen orientation.

In the polarimetry of the reflected light, the blue-pass filter was removed, and a feeble fundamental light reflected at the HS was used as an analytical light. We moved the half-wave plate to the position between the HS and the lens (L1) to eliminate the large anisotropy in the HS (±31%) induced by reflection of the polarization-rotating 800-nm light. Furthermore, we have to consider a difference in the sampling volume between the linear (reflected and transmitted fundamental light) and the nonlinear optical methods (SHG and TPEAF). The latter can limit the probing volume in the vicinity of the focused light spot owing to nonlinear optical processes, whereas the former probes a larger volume in front of and behind the focused spot. Multiple scattering in the sample deteriorates the focusing condition, resulting in a further increase in the sampling volume. The performance of the four polarimetric methods can be compared quantitatively by use of an equivalent sampling volume. To realize the comparison, we introduced confocal configuration with a 50-μm diameter pinhole in front of the analyzer, resulting in a transverse spot of 53 μm and an axial spot of 414 μm in air. The resultant radar graph showed a circular shape and no correlation between the graph shape and the collagen orientation [see Fig. 3(a)]. Although this result may reflect an inherent birefringence (0.003) in the collagen fiber[20] and/or surface scattering in the sample, it is too small to detect an appreciable difference between the two graph profiles with the present method.

In the polarimetry of the transmitted light, we used a similar confocal configuration to adjust the sampling volume. As a result, we observed similar crossed shapes in the vertical and horizontal orientations [see Fig. 3(b)]. This profile was oriented at 45 deg by rotating the sample to the same degree (not shown), resulting in a possible relation between the polarization direction and the collagen orientation. However, it is difficult to distinguish between the vertical and the horizontal orientations by use of this method, in which birefringence, absorption anisotropy, and/or forwardscattering in the sample could cause such a profile. Considering the very small birefringence in the collagen fiber and the low absorption in the infrared region, we conclude that the crossed profiles are due mainly to forward-scattering light process in the tissue.

In the TPEAF polarimetry, the center wavelength of the incident laser was changed to 730 nm to produce an efficient two-photon excitation. The TPEAF with a 425-nm emission maximum was observed from the human Achilles tendon at an excitation wavelength of 730 nm. The resultant TPEAF radar graph indicated that the major axis of the ellipsoid profile was consistent with the collagen orientation [see Fig. 3(c)]. However, it was less sensitive to the collagen orientation because of a small difference between the major and the minor axes of the ellipsoid. We believe that this property is due to the possible polarization dependence of the fluorescence quantum efficiency in the two-photon excited fluorophores. The major components of the human Achilles tendon are collagen and the elastin fibers. Both components show a blue autofluorescence under UV excitation. Possible fluorophores of the collagen and elastin are modified amino acids and pyridinoline aggregates, respectively. We observed weak fluorescence with an emission peak at 385 nm when irradiating the collagen powder with a UV line of 335 nm. On the other hand, the elastin powder and elastin fiber in human skin exhibit a 480-nm emission maximum.[21] However, when we observed the fluorescence of the mixture of collagen and elastin, the fluorescence of these components could not be distinguished from each other because of the spectral overlap of emis-

sion. From these results, we conclude that the TPEAF from the human Achilles tendon is caused by the mixture of collagen and elastin. Therefore the TPEAF polarimetry does not necessarily provide direct information about the collagen orientation, but it does provide indirect information about the local environments of collagen and elastin.

In the SHG polarimetry we observed a typical figure-eight shape with a marked axis parallel to the collagen orientation [see Fig. 3(d)]. This shape is characteristic of the uniaxial fiber orientation. Thus the SHG radar graph reveals the direction of the absolute orientation and the degree of the organization from the polarization angle of the major axis and the profile shape, respectively. The SHG process is caused by the second nonlinear polarization (P_2) as follows:

$$P_2 = \chi^{(2)} E^2, \tag{1}$$

where $\chi^{(2)}$ is the second-order susceptibility tensor and E is the electric field vector. In a microscopic region of a molecular order, $\chi^{(2)}$ is modified with the first hyperpolarizability β as follows[22]:

$$\chi^{(2)} = N_s \langle \beta \rangle, \tag{2}$$

where N_s is density of the molecules and $<\beta>$ is an average of the molecule direction with regard to β. Hence the collagen-induced SHG light depends on both the amount and the orientation of collagen molecules. It is important to note that the SHG polarimetry is effective in extracting the angular distribution of $<\beta>$ that is related to the collagen orientation. Hence, the SHG polarimetry offers a sensitive probe for the detailed distribution of the collagen orientation.

A comparison of the four kinds of probing lights by use of simple polarization optics indicated that SHG light was more sensitive to the collagen orientation than the other lights were. We emphasize that, although the other probing lights (autofluorescence and reflected and transmitted fundamental light) show interesting results when they are applied to other methods of comparison, they are substantially influenced by both collagen fibers and other tissue components (e.g., elastin and NADH for autofluorescence), resulting in contaminated data in the actual tissue. In contrast, collagen is SHG-only source in the tissues, giving it a specific sensitivity to the collagen orientation. Hence we can conclude that SHG light offers a superior performance in determining the collagen orientation.

C. Comparison of the Collagen Orientation in Biological Tissues

We compared SHG radar graphs among several tissue samples to evaluate the performance of SHG polarimetry. The figure-eight shape in the human Achilles tendon is characteristic of the uniaxial orientation of collagen fibers, as shown in Fig. 3(d). The resultant profiles of six other tissue samples are shown in Fig. 4. The gray arrows in each graph

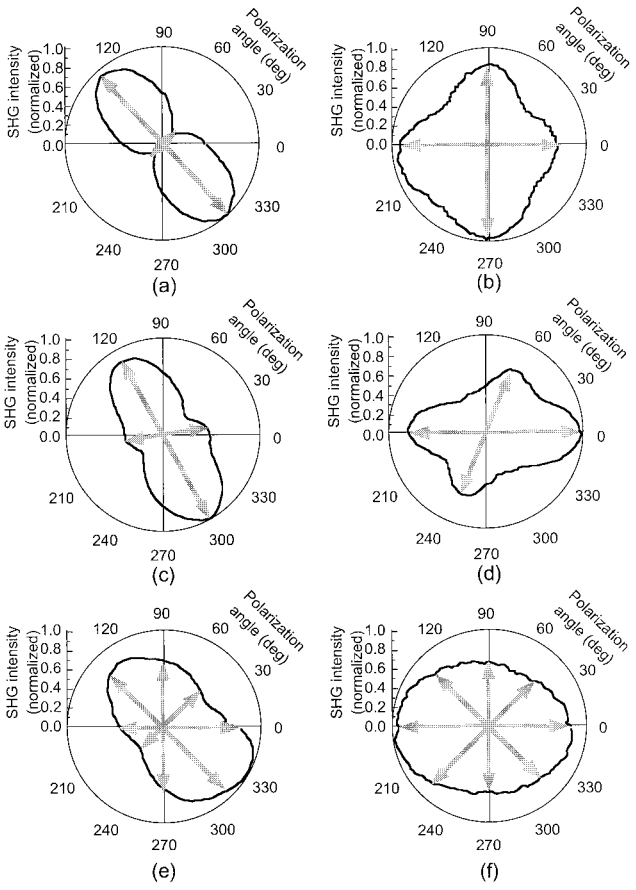

Fig. 4. SHG radar graphs for human tissue samples: (a) human dermis, (b) human dentin, (c) and (d) human talus, (e) chicken skin, and (f) collagen sponge. Gray arrows in each graph indicate the expected direction of the collagen orientation.

indicate the expected direction of the collagen orientation. There are characteristic differences among the samples owing to their corresponding collagen structures. The profile of the human dermis is similar to that of the human Achilles tendon; however, its figure-eight profile lacks a node [see Fig. 4(a)]. This result indicates that, unlike the fibers in the Achilles tendon, the orientation of collagen fibers in the human dermis is nearly, rather than completely, uniaxial. In general the anatomical results from the human dermis indicate that it possesses a tangled structure of collagen fibers that extends in every direction. We believe this that tangled structure of collagen fibers within the probing volume causes the imperfect figure-eight profile observed in the SHG polarimetry. The collagen fibers that exist in the form of a mesh structure in the dentin tissue result in a biaxial profile [see Fig. 4(b)]. However, the shape of the biaxial profile was distorted when it was measured from the dentin root toward the dentin crown (discussed in Section 5). In the human talus, we observed incompletely crossed biaxial shapes on different analytical spots [see Figs. 4(c) and 4(d)]. The acute angles between the two orientation axes (gray arrows) were similar to each other (=70 deg); however, the ratio of the length of the two orientation

axes was significantly different, depending on the analytical spot: 2.2:1, as shown in Fig. 4(c), and 1.3:1, as shown in Fig. 4(d). Such a difference may reflect the position-dependent distribution of the mechanical strength of the talus to the mechanical load against the talus. The result of the chicken skin sample reveals a distorted random collagen orientation because the specific axis is not apparent [see Fig. 4(e)]. The oval profile from the collagen sponge implies a completely random orientation of the collagen fibers [see Fig. 4(f)]. This observed diversity in the collagen orientation plays an important role in determining the original property of each tissue.

5. Distribution of the Collagen Orientation

An investigation of the localization or inhomogeneity of the collagen orientation provides important information for the study of tissue morphology and physiology. Here, as a fundamental study of the distribution of the collagen orientation, we measured the collagen orientation on the human dentin section at different analytical spots. Figure 5(a) shows a sketch of a cross section of a human tooth, on which four analytical spots are indicated, together with a growth line on the dentin tissue. The growth line, likened to the annual ring of a tree, indicates the age of the dentin tissue, which grows toward the crown of the tooth. By paying attention to this growth line and by examining the four corresponding SHG radar graphs [see Fig. 5(b)], we were able to formulate an interesting hypothesis. The resultant graphs for spots (1) and (2) are almost identical to each other. These two spots are considered to be on the same growth line and hence are formed simultaneously. On the other hand, the graphs for spots (3) and (4) are on different growth lines and show profiles that are different from those for (1) and (2). The spots that are on the same growth line may show the same metabolic characteristics. From a comparison of these graphs, we note that the shape of the radar graph is distorted from the root toward the crown, indicating that the orientation of the dentin collagen is disordered during tooth aging. Such a gradual disordering of the collagen orientation might be related to the metabolism of the dentin tissue. The results of nanosecond fluorescence measurement suggest that the aging mechanism for the crown dentin differs from that for the root dentin.[23] However, further examination of different types of teeth is necessary in order to confirm our hypothesis.

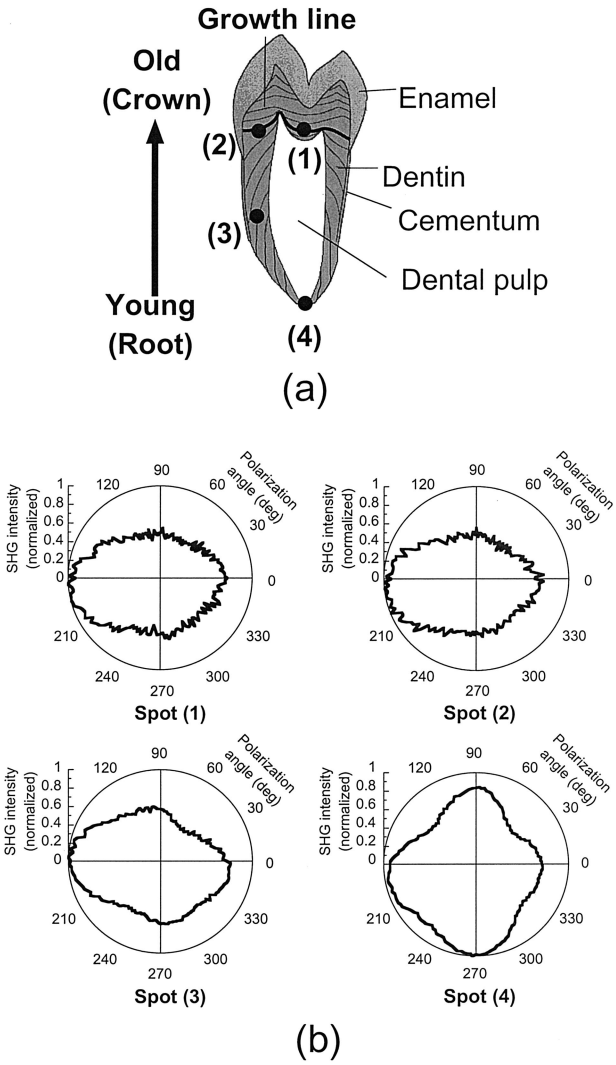

Fig. 5. Distribution of the collagen orientation in human dentin. (a) Sketch of a cross section of a tooth. The analytical spots and growth line in the dentin tissue are shown. (b) Results of the SHG polarimetry on four analytical spots.

6. Conclusion

We have proposed a method, based on reflection-type SHG polarimetry, for determining the collagen orientation in human tissues taken from a cadaver. The resultant SHG radar graphs reveal the direction of the absolute orientation and the degree of organization of collagen fibers in the tissues. From a comparison among the possible polarization measurements, we confirmed that the SHG polarimetry shows an excellent probing sensitivity to the collagen orientation. The apparent difference in the collagen orientation among several human tissues was demonstrated: a figure-eight shape for the human Achilles tendon and dermis, a biaxial shape for the dentin and talus, and an oval shape for the collagen sponge. Furthermore, we observed a position-dependent change in the collagen orientation in human dentin. From these findings we conclude that the proposed measurement system is a powerful tool for the biological research and clinical diagnosis of the change in the collagen fiber orientation in human tissues. In the near future we will apply the proposed measurement system to living human tissues.

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