Motion-artifact-robust, polarization-resolved second-harmonic-generation microscopy based on rapid polarization switching with electro-optic Pockells cell and its application to in vivo visualization of collagen fiber orientation in human facial skin

Yuji Tanaka,1 Eiji Hase,2 Shuichiro Fukushima,1,3 Yuki Ogura,4 Toyonobu Yamashita,4 Tetsuji Hirao,4 Tsutomu Araki,1 and Takeshi Yasui1,3,5,*

1Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama, Toyonaka, Osaka 560-8531, Japan
2Graduate School of Advanced Technology and Science, The University of Tokushima, 2-1 Minami-Josanjima, Tokushima 770-8506, Japan
3Department of Anatomy and Cell Biology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan
4Shiseido Research Center, 2-2-1 Hayabuchi, Tsuzuki-Ku, Yokohama 224-8558, Japan
5Institute of Technology and Science, The University of Tokushima, 2-1 Minami-Josanjima, Tokushima 770-8506, Japan
*yasui.takeshi@tokushima-u.ac.jp

Abstract: Polarization-resolved second-harmonic-generation (PR-SHG) microscopy is a powerful tool for investigating collagen fiber orientation quantitatively with low invasiveness. However, the waiting time for the mechanical polarization rotation makes it too sensitive to motion artifacts and hence has hampered its use in various applications in vivo. In the work described in this article, we constructed a motion-artifact-robust, PR-SHG microscope based on rapid polarization switching at every pixel with an electro-optic Pockells cell (PC) in synchronization with step-wise raster scanning of the focus spot and alternate data acquisition of a vertical-polarization-resolved SHG signal and a horizontal-polarization-resolved one. The constructed PC-based PR-SHG microscope enabled us to visualize orientation mapping of dermal collagen fiber in human facial skin in vivo without the influence of motion artifacts. Furthermore, it implied the location and/or age dependence of the collagen fiber orientation in human facial skin. The robustness to motion artifacts in the collagen orientation measurement will expand the application scope of SHG microscopy in dermatology and collagen-related fields.

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1. Introduction

Second-harmonic-generation (SHG) is a nonlinear optical phenomenon resulting from the nonlinear interaction of a high-peak-power optical electric field with a material [1] and has been widely used for wavelength conversion of laser light with a nonlinear optical crystal. On the other hand, when ultrashort pulse light is focused onto biological tissue instead of a crystal, SHG enables unique nonlinear optical microscopy having selectivity specific to non-centrosymmetric structures in biomolecules, such as collagen, myosin, and microtubules [2–4]. Furthermore, a contrast mechanism used in SHG microscopy is based on a naturally endogenous SHG process inherent in these molecules themselves, making it possible to visualize them in vivo freely from photobleaching, phototoxicity and additional staining with a fluorochrome. In particular, the collagen molecule is an important structural protein in the human body and forms structural aggregates successively, i.e., microfibrils, fibrils, fibers, and bundles of different sizes in biological tissues. Therefore, in vivo imaging of these collagen aggregates in tissues is an important biological application of SHG microscopy [5–12] because it is difficult to visualize collagen in vivo using conventional methods, including histological methods.

When biological tissue is likened to a skyscraper, collagen fibers play the role of reinforcing bars, whereas cells function as concrete. Therefore, the orientation of collagen fibers is closely related to the structural and functional properties of the tissues. For this reason, there is a considerable need for a reasonable method that can reveal the collagen orientation quantitatively in vivo in biological studies and clinical medicine. The efficiency of SHG light is sensitive to collagen orientation when the incident light is linearly polarized, and hence, polarization-resolved SHG (PR-SHG) microscopy is effective in visualizing the collagen fiber orientation in tissue quantitatively [13–20]. For example, the PR-SHG microscopy revealed ex vivo that a pattern of variation in collagen fibril orientation was different between normal and diseased cartilage [18]. Also, ex vivo PR-SHG imaging of photo aged mouse skin with deep wrinkles was demonstrated for investigating the relation between collagen fiber orientation and wrinkling direction, indicating that the wrinkling direction was significantly parallel to collagen orientation [19]. Furthermore, in vivo structural imaging in anesthetized rat’s cornea was successively achieved with PR-SHG microscopy in a reflection configuration [20]. However, there have been no attempts to perform in vivo PR-SHG imaging without the help of anesthesia.

PR-SHG imaging has to be performed by acquiring successive SHG images while changing the polarization angle of the incident laser light. Usually, the polarization angle is adjusted by mechanical rotation of a half-wave plate [13, 15–20]. However, such mechanical rotation requires a long waiting time and thus hinders achievement of in vivo PR-SHG imaging, because of unwanted motion artifacts caused by heart beats, breathing, and/or mechanical vibrations of the subjects. Although an electro-optic Pockells cell (PC) enables fast rotation of the laser light polarization in PR-SHG microscopy [14], it is difficult to achieve rapid image acquisition for in vivo applications because the required lock-in amplifier cannot be used with a highly sensitive photon-counting photomultiplier. In the work described in this article, by rapidly switching the polarization angle between vertical and horizontal directions at every pixel with a PC, we successively demonstrated in vivo visualization of collagen fiber orientation in human skin without the influence of motion artifacts.

2. Experimental methods

2.1 PR-SHG imaging based on two orthogonal polarizations

PR-SHG microscopy has two operation modes, depending on the method of polarization change. The first mode provides a radar graph of SHG light intensity or corresponding intensity profile with respect to the polarization angle by rotating the linear polarization angle continuously [13–18, 20]. The resulting SHG radar graph indicates detailed information of the
dominant orientation angle and the degree of fiber alignment. On the other hand, the second mode is based on the contrast of two SHG light beams when switching the laser polarization between the vertical and horizontal directions \[17, 19\]. Since the second mode involves simpler adjustment of the polarization angle than the first mode and is effective for a simple analysis of uniaxially oriented collagen fiber, we considered extending the second mode to \textit{in vivo} applications in this work. To evaluate the collagen orientation quantitatively in the second mode, the polarization anisotropy of SHG light (\(\alpha\)) was defined as follows

\[
\alpha = \frac{I_v - I_H}{I_v + I_H}
\]  

(1)

where \(I_v\) and \(I_H\) are the SHG intensities when the incident light is vertically and horizontally polarized, respectively. Then, the \(\alpha\) image was calculated by substituting \(I_v\) and \(I_H\) values at each pixel of two PR-SHG images for Eq. (1). The \(\alpha\) image reflects the distribution of collagen fiber orientation because strong SHG light is observed when laser polarization is parallel to the collagen orientation, and SHG light is considerably weaker if the laser polarization is perpendicular to the collagen orientation. The collagen orientation is uniaxial for \(\alpha = \pm 1\) and random or biaxial for \(\alpha = 0\). The sign of \(\alpha\) gives the dominant direction of the collagen orientation: positive for a vertical orientation and negative for a horizontal orientation.

The previous measurement procedure in the second mode is as follows: (1) one PR-SHG image is acquired under vertical polarization of the incident laser light (vertical-polarization-resolved SHG image or VPR-SHG image), (2) the polarization angle of the laser light is changed from the vertical to the horizontal direction by mechanical rotation of a half-wave plate, and (3) another PR-SHG image is acquired under horizontal polarization of the incident laser (horizontal-polarization-resolved SHG image or HPR-SHG image), as shown in Fig. 1(a) \[17, 19\]. Therefore, the VPR-SHG image is composed of the former half of the data acquisition numbers ( = 1, 2, 3, \ldots, \(m-2, m-1, m\)), whereas the HPR-SHG image is composed of the latter half of them ( = \(m+1, m+2, m+3, \ldots, 2m-2, 2m-1, 2m\)). In this case, a data acquisition time ranging from subseconds to a few seconds is required for the steps (1) and (3), whereas step (2) needs a waiting time of about several seconds depending on the speed of mechanical rotation. Such serial measurement of two orthogonal PR-SHG images interrupted by the mechanical polarization rotation is highly sensitive to the unwanted motion artifacts, making it difficult to satisfy a pixel-to-pixel correspondence between those two PR-SHG images.

To solve the above problem, we should consider speeding up the polarization switching and improving the data acquisition method. The polarization switching can be speeded up to a few tens MHz by using a PC. Regarding the improvement of the data acquisition method, a VPR-SHG signal and an HPR-SHG signal should be acquired alternately at every pixel, as shown in Fig. 1(b). In this case, the VPR-SHG image is composed of odd data acquisition numbers ( = 1, 3, 5, \ldots, \(2m-5, 2m-3, 2m-1\)), whereas the HPR-SHG image is composed of even data acquisition numbers ( = 2, 4, 6, \ldots, \(2m-4, 2m-2, 2m\)). Since the polarization-switching rate by the PC is much faster than the motion artifacts, it is easy to satisfy a pixel-to-pixel correspondence between the VPR-SHG and HPR-SHG images. Therefore, a combination of the rapid polarization switching by PC and step-wise raster scanning by galvanometer mirror (GM) will enable us to obtain an \(\alpha\) image correctly without motion artifacts.
2.2. Experimental setup

Figure 2(a) shows the experimental setup. The developed PR-SHG microscope was equipped with a mode-locked Cr:Forsterite laser (Avesta Project Ltd., CrF-65P, center wavelength = 1250 nm, pulse duration = 70 fs, repetition rate = 73 MHz) for a laser source that reduces the laser-induced photodamage [21–23] and enhances the penetration depth [8]. We used a PC made of lithium tantalate crystal (Conoptics Inc., 360-120, thickness = 120 mm, half-wave voltage = 160 V at 1250 nm, contrast ratio = 100:1, refractive index = 2.13 at 1250 nm, group velocity dispersion = 144 fs²/mm), driven by a digital amplifier (Conoptics Inc., 25D, bandwidth < 30 MHz, maximum voltage = 175 V), for rapid switching of two orthogonal polarizations. To suppress the temporal broadening of the pulse duration in the PC, we prechirped the laser light using a pair of dispersion prisms made of S-NPH3 glass (Ohara Inc., refractive index = 1.90 at 1250 nm, group velocity dispersion = −30 fs²/nm) before passing through PC. Figure 2(b) compares the autocorrelation traces of the laser light before and after passing through the prism pair and PC. The adjusted negative dispersion in the prism pair cancels the positive dispersion in the PC, making it possible to maintain the original pulse duration of this laser source after passing through the prism pair and PC. A half-wave plate (Lattice Electro Optics Inc., CWO-1250-02-10, retardation tolerance = λ/500), set into a
stepping-motor-driven rotatory mount (Suruga Seiki Co., Ltd., K491-30, angle resolution = 0.004 deg/pulse, driving pulse frequency = 1.875 kHz, rotating speed = 7.5 deg/s), after PC was used for the mechanical-rotation-based PR-SHG imaging described later. After switching the polarization between the vertical and horizontal directions with the PC, the focus spot of the laser beam is scanned two-dimensionally onto a sample by use of a pair of GMs, composed of a fast GM (scanning freq. = 64 Hz) and a slow GM (scanning freq. = 0.5 Hz), a pair of relay lenses, and an objective lens (OL; Nikon Instruments Inc., CFI Plan 50 × H, magnification = 50, NA = 0.9, working distance = 350 μm, oil-immersion type). To cancel the polarization dependence of the reflectivity in the GM, the vertical and horizontal polarizations of the laser light were respectively set at a direction of 45 degree and −45 degree from the p-polarization in the fast GM or the s-polarization in the slow GM. The average power of the laser light was set to be 28–35 mW on the sample. Backscattered SHG light was collected via the objective lens and then was separated from the laser light by a harmonic separator (Lattice Electro Optics Inc., LWP-45-R, 625-T, 1250-B-1013, reflected wavelength = 625 nm) and an optical filter with a sharp pass-band (Semrock Inc., 625/26 nm BrightLine, pass wavelength = 612 to 638 nm). Finally, the SHG light was detected by a photon-counting photomultiplier with Peltier cooling (PMT; Hamamatsu, H7421-40) connected with a pulse counter. We used a customized attachment ring [see an inset of Fig. 2(a)] for in vivo imaging of human facial skin, which is attached to the subject’s skin by a double-stick tape and used to reduce the motion of its measured position. Figure 2(c) shows a photograph of in vivo measurement of the human facial skin.

To correctly perform the data acquisition procedure in Fig. 1(b), scanning signals of the slow GM and fast GM, a switching signal of the PC, and a timing signal of the data acquisition should be synchronized. To this end, we used a timing chart for these four signals as shown in Fig. 2(d). First, the slow GM was scanned at 0.5 Hz with a step-wise waveform (number of steps = 128, time duration of one step = 15.6 ms). Second, the fast GM was scanned at 64 Hz with a step-wise waveform (number of steps = 128, time duration of one step = 122 μs). Third, the PC was driven by a 8,192-Hz square wave swinging between the half-wave voltage ( = 160 V, corresponding polarization direction = vertical) and the no-bias voltage ( = 0 V, corresponding polarization direction = horizontal) because the polarization angle has to be changed from the vertical to the horizontal direction during one step of the step-wise waveform for the fast GM in order to switch the polarization at the same pixel. Fourthly, the timing signal of the data acquisition was set to be twice as high frequency ( = 16,384 Hz) as the switching signal of the PC. Finally, the serial data train of SHG signals (data acquisition number = 1, 2, 3, ..., 2m-2, 2m-1, 2m = 1, 2, 3, ..., 65534, 65535, 65536) was acquired by a counter board in a computer and sorted into the VPR-SHG image (data acquisition number = 1, 3, 5, ..., 2m-5, 2m-3, 2m-1 = 1, 3, 5, ..., 65531, 65533, 65535) and the HPR-SHG image (data acquisition number = 2, 4, 6, ..., 2m-4, 2m-2, 2m = 2, 4, 6, ..., 65532, 65534, 65536). The two scanning signals of the fast GM and slow GM, the switching signal of the PC, and the timing signal of the data acquisition were provided from two signal generators (NF Corporation, WF1974), synchronizing to each other. We confirmed the synchronization of all signals by monitoring the two angle-sensor signals of GMs, the two optical signals separated by a polarization beamsplitter after passing through PC, and data acquisition signal from the signal generator simultaneously in another setup (not shown). In this way, one can obtain the α image, satisfying a pixel-to-pixel correspondence between VPR-SHG and HPR-SHG images even though the motion artifacts exist.
Fig. 2. (a) Experimental setup. PC: Electro-optic Pockells cell; PMT: photon-counting photomultiplier with Peltier cooling. Inset is a photograph of the attachment ring. (b) Comparison of autocorrelation traces of the laser light before and after passing through the prism pair and PC. (c) Photograph of in vivo measurement of the human facial skin. (d) Timing chart of scanning signals of slow GM and fast GM, switching signal of PC, and timing signal of data acquisition.
2.3 Sample preparation

We prepared the human Achilles tendon as the sliced specimen for ex vivo PR-SHG imaging. The human Achilles possesses the uniaxial orientation of collagen fiber along the axial direction. The human Achilles was sliced to 1 mm thick along the axial direction. After washing the sliced specimen with distilled water, the specimen was put between a cover slip and a glass slide to flatten the sample surface.

3. Results

3.1 Ex vivo PR-SHG imaging of human Achilles tendon with uniaxial collagen orientation

To evaluate the correctness of the collagen orientation analysis using the PC-based PR-SHG microscopy, we first performed ex vivo PR-SHG imaging of a sliced specimen of the human Achilles tendon. Figure 3 shows the VPR-SHG image, the HPR-SHG image, and an \( \alpha \) image when the collagen orientation of the sample was directed at (a) 90 degree (vertical orientation), (b) 0 degree (horizontal orientation), and (c) 45 degree (middle between horizontal and vertical orientations), respectively. The VPR-SHG image, HPR-SHG image, and \( \alpha \) image with a size of 300 µm × 300 µm, composed of 128 × 128 pixels, were measured in an image acquisition time of 2 s (1 s for VPR-SHG imaging and 1 s for HPR-SHG imaging). For comparison, we also performed similar imaging of the same specimen with the previous PR-SHG microscopy based on the mechanical rotation of a half-wave plate, as shown in Fig. 4. In this case, an image acquisition time of 8 s (1 s for VPR-SHG imaging, 6 s for the mechanical rotation, and 1 s for HPR-SHG imaging) was required for these three kinds of image (image size = 300 µm × 300 µm, pixel size = 128 × 128 pixels). In Figs. 3 and 4, although it is difficult to find a large difference between the VPR-SHG and HPR-SHG images, \( \alpha \) images clearly revealed the difference in the sample direction, or collagen orientation. For example, the \( \alpha \) image in Figs. 3(a) and 4(a) shows a bluish distribution, indicating that the vertical collagen orientation is dominant. On the other hand, the reddish \( \alpha \) image in Figs. 3(b) and 4(b) shows that the collagen fiber is mainly orientated in the horizontal direction. Furthermore, the whitish \( \alpha \) image in Figs. 3(c) and 4(c) reveals that the \( \alpha \) values of vertical and horizontal orientations were cancelled due to the collagen orientation of 45 degree. Good agreement of the \( \alpha \) image color between Figs. 3 and 4 clearly indicated that the developed system could measure the collagen orientation correctly ex vivo in the same manner as the previous PR-SHG microscopy.
Fig. 3. VPR-SHG image, HPR-SHG image, and $\alpha$ image of human Achilles tendon ex vivo measured by the PC-based PR-SHG microscopy (image size = 300 µm × 300 µm, pixel size = 128 pixel × 128 pixel, image acquisition time = 2 s). The collagen orientation of the sample was directed at (a) 90 degree (vertical orientation), (b) 0 degree (horizontal orientation), and (c) 45 degree (middle between horizontal and vertical orientations), respectively.
3.2 In vivo PR-SHG imaging of dermal collagen fiber in human facial skin

Two Japanese male volunteers aged 24 years old and 57 years old were recruited for in vivo imaging of the facial skin. Based on the previous study in which the risk of laser-induced photodamage to human skin was investigated [11], we set the average power of the laser light radiated onto the skin to 35 mW. Written informed consent was obtained from all subjects before the measurement. The protocol conformed to the Helsinki Declaration and was approved by the ethics committees for human experiments in both Osaka University and Shiseido Co., Ltd.

To evaluate the robustness to motion artifacts, we compared α images of the cheek skin of a male subject in his 24 years old continuously acquired with the PC-based PR-SHG microscopy and the mechanical-rotation-based PR-SHG microscopy. Figure 5 shows a series of α images (image size = 400 µm by 400 µm region, pixel size = 128 pixels × 128 pixels) acquired every 2 s (1 s for VPR-SHG imaging and 1 s for HPR-SHG imaging) at the same region in the cheek skin using the PC-based PR-SHG microscopy. Also, Media 1 shows a corresponding movie of a series of continuously acquired VPR-SHG images, HPR-SHG images, and α images. Pores in the skin appeared as a black circle in those images because of no collagen content. Although the captured images fluctuated due to the motion artifacts in the subject, a little bluish tone and its distribution were maintained in the same way for all α images. This result is evidence that there is a pixel-to-pixel correspondence between the VPR-SHG image and the HPR-SHG image under the motion artifacts. On the other hand, α images...
obtained with the mechanical-rotation-based PR-SHG microscopy made a striking contrast to those obtained with the PC-based PR-SHG microscopy. Figure 6 shows a series of $\alpha$ images (image size = 400 µm by 400 µm region, pixel size = 256 pixels × 256 pixels) acquired every 10 s (2 s for VPR-SHG imaging, 6 s for the mechanical rotation, and 2 s for HPR-SHG imaging) at the same region in the cheek skin of the same subject using the mechanical-rotation-based PR-SHG microscopy, and Media 2 shows a corresponding movie for a series of continuously acquired VPR-SHG images, HPR-SHG images, and $\alpha$ images. The captured images in Fig. 6 and Media 2 also fluctuated due to the motion artifacts in the same manner as that in Fig. 5 and Media 1. However, the behavior of $\alpha$ images is quite different from that in the PC-based PR-SHG microscopy. That is to say, the tone of the $\alpha$ image largely varies between bluish and reddish colors every frame of the $\alpha$ image. This is because the waiting time for the mechanical rotation ($=6$ s) makes it difficult to keep the pixel-to-pixel correspondence between the VPR-SHG image and the HPR-SHG image, resulting in incorrect $\alpha$ images due to pixel mismatching between them. Considering the comparison between Figs. 5 and 6, it should be noted that the data from two different methods were compared with different time acquisition. However, since the SHG microscopy was constructed firmly and the customized attachment ring was used to suppress the motion artifacts of the measured position in the human facial skin, the effect of microscope drift is negligible. Furthermore, although the total image acquisition time ($=10$ s) can be reduced by the fast motor or liquid crystal retarder, we consider that it is still difficult even for these methods to satisfy a pixel-to-pixel correspondence between two PR-SHG images due to the motion artifacts. A comparison of $\alpha$ images between Figs. 5 and 6, or Media 1 and Media 2, underlines the clear advantage of the PC-based PR-SHG microscopy over the mechanical-rotation-based PR-SHG microscopy regarding the motion artifacts.

Fig. 5. A series of $\alpha$ images (image size = 400 µm by 400 µm region, pixel size = 128 pixels × 128 pixels) acquired every 2 s using the PC-based PR-SHG microscopy (Media 1). The same region in the cheek skin for a male subject in his 24 years old was continuously measured.
Finally, to evaluate the potential for \textit{in vivo} applications in the dermatology, we applied the PC-based PR-SHG microscopy to visualize the distribution of dermal collagen fiber orientation in human facial skin \textit{in vivo}. We selected two portions of the facial skin for measurement: cheek skin and eye corner skin. To enlarge the imaging region, we acquired an SHG image of a 400 µm by 400 µm regions by using the GM and successively acquired the neighboring regions after scanning the sample position by 400 µm by using a stepping-motor-driven translation stage as shown in Fig. 2(a) [11, 12]. We obtained a large SHG image with a size of 1.6 mm by 1.6 mm by arranging the 16 acquired SHG images in a matrix of four rows and four lines. Figures 7(a) and 7(b) respectively show \(\alpha\) images in the cheek skin and the eye corner skin for a male subject in his 24 years old. A mixture of bluish and reddish colors in Fig. 7(a) indicated that the cheek skin has a neutral distribution of the vertical and horizontal orientation of the collagen fibers. By contrast, the \(\alpha\) image in Fig. 7(b) became more reddish than that in Fig. 7(a), indicating that the horizontal orientation of the collagen fiber is dominant in the eye corner skin. We also performed a similar measurement for a male subject in his 57 years old, as shown in Figs. 7(c) and 7(d). The resulting \(\alpha\) images are similar to those in the male subject in his 24 years old, indicating the mixed distribution of vertical and horizontal collagen orientation in his cheek skin and the predominant distribution of horizontal orientation in his eye corner skin. However, bluish color in Fig. 7(a) and reddish color in Fig. 7(b) were enhanced in Figs. 7(c) and 7(d). We will discuss the location and/or age dependence of the collagen fiber orientation in human facial skin later.
4. Discussion

We applied the rapid polarization switching by the PC to the second mode in the PR-SHG imaging based on two orthogonal polarizations. This mode is useful for the simple analysis of uniaxial collagen orientation, such as wrinkled skin or tendon. However, it is difficult for this mode to evaluate the complicated collagen orientation because the $\alpha$ image involves both the dominant orientation angle and the degree of fiber alignment. For example, $\alpha$ image is not directly related with the angle image of collagen orientation. Also, the second mode cannot distinguish the random orientation and biaxial orientation because both reveal $\alpha = 0$. For more detailed analysis of collagen fiber orientation, the first mode of PR-SHG imaging based on continuously rotating polarization will be more powerful. We here discuss a possibility to extend our PC-based PR-SHG microscopy to the first mode. Although the two orthogonal polarizations for the second mode were switched by applying the square-wave signal swinging between 0 V and the half-wave voltage to the PC, the polarization angle can be also rotated continuously by applying a ramp-wave signal ranging from 0 V to half-wave voltage to the PC in combination with a quarter-wave plate [14]. Therefore, if a series of PR-SHG signal is continuously acquired with respect to a full or half rotation of polarization angle at every pixel, the first mode will also become robust to the motion artifacts and hence will be used for various applications in vivo. Since our PC can operate 1,000-times faster than the switching frequency demonstrated above, it is easy to make the full or half rotation of the polarization angle during one step of the step-wise waveform for the fast GM. Therefore, it should be possible to extend the PC-based PR-SHG microscopy to the first mode in vivo for the detailed analysis of the complicated collagen orientation.

It will be interesting to compare the PR-SHG imaging to another SHG imaging without the need for the polarization rotation because both methods are robust to the motion artifacts in the imaging of the collagen fiber orientation. Recently, the analysis of the collagen fiber orientation in tissues has been achieved based on two-dimensional discrete Fourier transform (2D-FT) of SHG image [24–26]. In this method, under incidence of a circularly polarized laser light, a single SHG image was obtained, and then 2D-FT analysis of the image was...
effectively performed for evaluation of collagen fiber orientation. Due to 2D-FT analysis, the calculation of the collagen fiber orientation has been done from a single SHG image without performing PR-SHG imaging. Although this method does not need the polarization rotation and hence can be performed by the simpler setup, SHG image has to be visualized at sufficiently high spatial resolution for correct image analysis with 2D-FT. In other word, this method cannot provide the information at the molecular level due to the insufficient spatial resolution. On the other hand, because the polarization dependence of SHG efficiency is arisen from the molecular axis, PR-SHG imaging will be useful for investigating the information at the molecular level, for example, the calculation of the ratio of the $\chi^{(2)}$ tensor elements [27].

We next discuss the location dependence of collagen fiber orientation in human facial skin. Regarding the 24-years-old male subject, a mixture of bluish and reddish colors in Fig. 7(a) indicated that the cheek skin has a neutral distribution of the vertical and horizontal orientation of the collagen fibers. On the other hand, the reddish $\alpha$ image in Fig. 7(b) revealed that the horizontal orientation of the collagen fiber is dominant in the eye corner skin. The reason why we selected the cheek skin and eye corner skin for measurement is in existence of skin wrinkles. Deep wrinkles are not formed in the cheek skin whereas they often appear as an extension of the eye corner on its skin. The wrinkle at the eye corner is mainly due to repeated mechanical loads of tension and compression on the skin when blinking the eyes. Previously, we have confirmed from ex vivo PR-SHG imaging of the wrinkled mouse skin that the wrinkle direction was parallel to the collagen orientation [19]. However, since this experiment was performed after deep wrinkles appeared, it is not clear which comes first, the change of the collagen orientation or the formation of the wrinkles. Since this 24-years-old subject has no wrinkles at the eye corner skin yet, the reddish $\alpha$ image in Fig. 7(b) may imply the fact that the change of the collagen orientation forms the wrinkles on the skin. Also, the degree of reddish color may forecast the possibility of wrinkles appearing in the future even though the wrinkles do not appear yet.

We also discuss the age dependence of the collagen fiber orientation by comparing $\alpha$ images between two male subjects in their 57 years old and 24 years old. Regarding the 57-years-old male subject, several wrinkles slightly appeared at his eye corner whereas a little sagging and nasolabial fold were observed on his cheek, in contrast to the 24-years-old subject. The $\alpha$ images for the 57-years-old subject [see Figs. 7(c) and 7(d)] were almost similar to those for the 24-years-old subject [see Figs. 7(a) and 7(b)], showing a neutral distribution of the vertical and horizontal orientation in the cheek skin and the horizontal orientation in the eye corner skin. Although the statistical analysis is essential for more precise analysis of the collagen fiber orientation in human skin, those preliminary results may imply that the location difference of the collagen fiber orientation is common regardless of the age. Next, we compared the $\alpha$ images of the same skin portion between different ages and confirmed that bluish color in Fig. 7(a) and reddish color in Fig. 7(b) were enhanced in Figs. 7(c) and 7(d), respectively. The enhanced reddish color in Fig. 7(d) will be related to several wrinkles slightly appearing at the eye corner in the 57-years-old male subject because the wrinkle direction was parallel to the collagen orientation ex vivo [19]. On the other hand, the increased bluish color in Fig. 7(c) may be caused by the sagging skin and/or the nasolabial fold because they were often observed along the vertical direction. Although the number of the subjects should be increased for statistical analysis to conclude the location and/or age dependence of the collagen fiber orientation in human facial skin, these preliminary results imply the potential of PC-based PR-SHG microscopy for in vivo assessment of collagen fiber orientation.

5. Conclusion

We constructed a PC-based PR-SHG microscope suitable for in vivo visualization of collagen fiber orientation in human skin. Robustness against motion artifacts was achieved by rapid
polarization switching at every pixel by using a PC. A comparison between the PC-based PR-SHG microscopy and the mechanical-rotation-based PR-SHG microscopy clearly highlighted its advantage in terms of robustness against motion artifacts. Furthermore, we applied the PC-based PR-SHG microscopy to in vivo imaging of collagen orientation in human facial skin. To the best of our knowledge, this is the first time that the orientation of dermal collagen fibers in human facial skin has been successively visualized in vivo. The visualized orientation of dermal collagen fibers was significantly different between the cheek skin and the eye corner skin, and also between the 24-years-old and 57-years-old subjects. PC-based PR-SHG microscopy will be a powerful tool for in vivo assessment of collagen fiber orientation in the field of dermatology and collagen-related fields, including ophthalmology [20] and orthopedics [18]. Furthermore, if this microscopy is combined with an endoscopy technique [10], its application field will be further extended to the clinical medicine. Also, it should be possible to employ this microscopy in various applications [28] in addition to collagen orientation.

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