Second Harmonic Generation Imaging Analysis of Collagen Arrangement in Human Cornea

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PURPOSE. To describe the horizontal arrangement of human corneal collagen bundles by using second harmonic generation (SHG) imaging.

METHODS. Human corneas were imaged with an inverted two photon excitation fluorescence microscope. The excitation laser (Ti:Sapphire) was tuned to 850 nm. Backscatter signals of SHG were collected through a 425/30-nm bandpass emission filter. Multiple, consecutive, and overlapping image stacks (z-stacks) were acquired to generate three dimensional data sets. ImageJ software was used to analyze the arrangement pattern (irregularity) of collagen bundles at each image plane.

RESULTS. Collagen bundles in the corneal lamellae demonstrated a complex layout merging and splitting within a single lamellar plane. The patterns were significantly different in the superficial and limbal cornea when compared with deep and central regions. Collagen bundles were smaller in the superficial layer and larger in deep lamellae.

CONCLUSIONS. By using SHG imaging, the horizontal arrangement of corneal collagen bundles was elucidated at different depths and focal regions of the human cornea.

Keywords: cornea, collagen, second harmonic generation, corneal stroma

Both the cornea and sclera have a similar bulk collagen content; however, differences in collagen type and their arrangement result in substantially different optical properties.¹ In corneal tissue, collagen fibrils (31–34-nm thick) form collagen bundles (5–35-μm thick), which are grouped into approximately 200 lamellae (1–2-μm thick and 10–200-μm wide) that lie roughly parallel to the surface of the globe.^{1–3} The narrow and uniform diameter and regular lateral packing of corneal collagen fibrils are considered as two main contributors to corneal transparency.¹

Historically, light microscopy, scanning electron microscopy, and x-ray diffraction technology have been used to study the lamellar arrangement in corneal collagen.¹ However, light or scanning electron microscopy require tissue processing including fixation and dehydration, which alters the normal three-dimensional (3D) configuration of collagen architecture. Although x-ray diffraction technology can detect collagen orientation in a large region, its use is limited as it cannot directly reveal minute architectural details.

Recently, multiphoton microscopy was introduced and has been used increasingly in laboratory-based biomedical imaging.⁴ Multiphoton microscopy includes two photon excitation fluorescence (TPEF) and second harmonic generation (SHG). The former involves autofluorescent process enabled through the absorption of two photons, while the latter is an absorption-free process. Imaging with SHG signals has emerged as a useful tool to evaluate corneal collagen organization and enables 3D analysis of the collagen architecture at the submicron level and confers several advantages over other imaging techniques such as confocal and electron microscopy.^{4,5} Fixatives and dyes are not necessary for SHG

imaging, and it is possible to image in vivo with minimal invasiveness. Moreover, near-infrared excitation wavelengths used in SHG imaging allows deeper imaging depth, greater than 1000 μm.⁶ However, there are drawbacks to SHG imaging including limited ability to image highly scattering tissue.⁴ Both backward and forward scatter SHG imaging has been introduced. Backward scatter SHG imaging is noninvasive but the signal intensity is lower compared with forward scatter SHG imaging.⁴

Intimate knowledge of collagen fiber orientation throughout the cornea not only enriches our understanding of its structural and mechanical properties, but is also crucial for continued development of the artificial cornea.⁷

Using the aforementioned advantages of SHG imaging, recent studies have detailed the sagittal (anterior to posterior) lamellar collagen fiber arrangement from limbus to limbus in the human cornea.^{5,8,9} These studies have enhanced our understanding of corneal biomechanics as it relates to various pathologic conditions.^{10,11} It has been shown that corneal collagen lamellae split and merge not only in the anterior-posterior direction, resulting in interlamellar connections, but also within the lamellar planes.¹² Therefore, a detailed study of the horizontal planar lamellar collagen fiber arrangement may prove useful.

In this study, serial high-resolution lamellar images of the human cornea were obtained by using SHG imaging. By analyzing the collagen arrangement in each image plane separated by 1 μm, horizontal collagen bundle arrangement in the corneal lamellae was investigated.

TABLE. Clinical Characteristics of Donor Corneal Tissue Used in This Study

Tissue No.	Age	Race	Sex	Laterality	Harvest to Imaging Time	Stromal Thickness
1	55	White	Female	OD	5 d	477
2	55	White	Female	OS	5 d	475
3	66	White	Male	OD	4 d	469
4	60	White	Female	OD	5 d	482
5	60	White	Female	OS	5 d	485
6	14	Black	Male	OD	11 d	480
7	14	Black	Male	OS	11 d	477

Stromal thickness was defined as the distance between the z planes where the first and the last SHG signal were detected at central corneal area.

MATERIALS AND METHODS

This study was approved by the institutional review board of the Albert Einstein College of Medicine, Yeshiva University (New York, NY, USA) and adhered to the tenets of Declaration of Helsinki. Seven eye bank corneas, which were not suitable for human transplantation were obtained from the Central Florida Lions Eye and Tissue Bank (Tampa, FL, USA).

Sample Preparation

Eye bank corneas (Table) were stored in Optisol GS (Bausch & Lomb, Rochester, NY, USA) solution until imaged. These corneas were classified as 'research only' because of various reasons (mild endothelial pleomorphism/polymegathism, diffuse moderate endothelial stress line, and guttata) but maintained in an optically transparent state. All imaging was completed within 12 days of harvesting corneal tissue. Corneas were first transferred to a glass-bottom dish (35 mm; MatTek, Ashland, MA, USA) and several drops of balanced salt solution (BSS; Alcon, Fort Worth, TX, USA) were applied to the samples

to prevent desiccation. To image the epithelial side, the sample corneal button was placed upside down, with the epithelium against the glass bottom. To image the endothelial side, corneal buttons were positioned right-side up, with the endothelium facing the glass bottom.

Second Harmonic Generation Imaging Process

Second harmonic generation imaging was performed using an inverted TPEF microscope (FluoView FV-1000; Olympus, Central Valley, PA, USA). The imaging process was as follows: briefly, the cornea was placed on the glass-bottom plate (35 mm; MatTek; Supplementary Fig. S1), a laser (Ti:Sapphire) was tuned to 850 nm and directed through a (RDM 690 nm) dichroic mirror, and a $\times 25$ (N.A. = 1.05) water immersion objective was used to focus the excitation beam and to collect backscatter signals; the SHG signal was collected through a bandpass emission filter (425/30 nm) after reflection by a dichroic mirror (dm458), a square image (513×513 - μm) was acquired with 1024×1024 pixels of resolution in approximately 15 seconds, and multiple, consecutive, and overlapping image stacks (z-stacks) were acquired using the same objective lens. When images were z-stacked, samples were scanned in 1- to 10- μm steps in the z-axis to generate 3D data sets. For reference, 0- μm position in depth corresponds to the first z-position where SHG signal is detected. ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) was used to analyze acquired images.

Image Analysis With ImageJ

Tracing the direction of individual collagen bundles from backward-scattered SHG images is much more challenging than from forward-scatter SHG images. Corneal collagen bundles changing directions cause corresponding changes in SHG images. To deconvolute and simplify indirect estimation of collagen arrangement, ImageJ software was used. Grayscale SHG images were processed by sequential application of image modifications. First, an image was processed by application of

FIGURE 1. Image processing by using ImageJ software is demonstrated. (A) The original SHG image was obtained from superficial lamella of human cornea (55-year-old white female). (B) The same image in (A) was processed by application of spatial frequency filters (ImageJ $\rightarrow$ Process $\rightarrow$ Fourier transform [FFT] $\rightarrow$ Bandpass filter). Details were simplified and contrast was enhanced. (C) The filtered image in (B) was converted to binary (ImageJ $\rightarrow$ Process $\rightarrow$ Binary $\rightarrow$ Make binary). This final image was used to analyze the complexity of collagen bundle arrangement. (D–F) Each black and white conversion along the vertical lines in (C) is plotted as a peak in the graphs (D) for line 1, (E) for line 2, and (F) for line 3. The number of peaks crossing the median cut-off value (dotted line) of signal intensity is similar regardless of measurement area and is counted 11 (D), 10 (E), and 11 (F), respectively.

FIGURE 2. The collagen bundle arrangement in corneal lamella was shown. (A) In this SHG image (at 80- μ m depth of the central cornea, 14-year-old black male), the collagen bundles show an irregular pattern of arrangement. (B) The orientations of collagen bundles are indicated by yellow lines. The curved arrangement of collagen bundles in the corneal lamellae made the capturing of a long collagen fiber difficult in this tangential image plane. Therefore, collagen fibers demonstrate a feather like appearance. In this anterior section of cornea, no preferred direction of collagen bundle was discerned.

spatial frequency filters (ImageJ $\rightarrow$ Process $\rightarrow$ Fourier transform (FFT) $\rightarrow$ Bandpass filter; Fig. 1). ImageJ bandpass filters have the capacity to remove both high and low spatial frequencies. The filter sets for large and small structures were set 100 and 20 pixels, respectively. Suppression stripes were set to 'none' and tolerance of direction was set at 5%. 'Autoscale after filtering' and 'saturate image when autoscaling' were activated during processing. Second, the filtered images were converted to binary (ImageJ $\rightarrow$ Process $\rightarrow$ Binary $\rightarrow$ Make binary). The crossing density of black and white areas in converted images were measured (ImageJ $\rightarrow$ analyze $\rightarrow$ gel $\rightarrow$ plot lanes) and defined as the number of peaks crossing median cut-off intensity. This method yielded relatively constant values regardless of measurement area in the same image. The measured crossing densities at vertical midline indirectly represent the complexity of collagen bundle patterns (Fig. 1). The higher the crossing density, the more complex the pattern is. Therefore, crossing density was designated as 'irregularity' in this study. Irregularity was compared along differing depths (superficial, anterior, middle, and posterior central cornea) and different regions (central, peripheral, and limbal area) of cornea. Considering the normal thickness discrepancy between central, peripheral, and limbal corneas, the anterior, middle, and posterior layers were measured at 10% (superficial), 25% (anterior), 50% (middle), and 75% (posterior) depths of the central cornea, temporal peripheral cornea (approximately 4 mm away from the corneal center), and at the temporal limbus. Measurements were taken from seven corneas and each plane was measured in triplicate and mean used for analysis.

Statistical Analysis

Statistical analysis was performed using SPSS software ver.20.0 (SPSS, Inc., Chicago, IL, USA). Normality of data was assessed by the Shapiro-Wilk test. Because the data did not follow a normal distribution, the Kruskal-Wallis test with Bonferroni correction was used to compare means of different groups. *P* values (two-tailed) less than 0.05 were considered significant.

RESULTS

Corneal Collagen Bundle Structure

Analysis of the collagen bundle unit revealed complex merging and splitting patterns within the corneal lamellae (Fig. 2,

Supplementary Videos S1–S3). These merging and splitting patterns displayed marked differences at different axial depths. Centrally, the superficial layer near Bowman's membrane showed the most irregular arrangement of collagen bundles. The size of each bundle was found to be smaller in the superficial than in the deep layers. The distribution of oblique collagen bundles attached to Bowman's membrane resulted in the characteristic "dotted" appearance of the superficial corneal lamellae (Fig. 3; Supplementary Video S1). In deeper central corneal layers, the collagen bundles were found to be larger and distributed in homogenous patterns (Supplementary Videos S2, S3).

Collagen bundle patterns also displayed marked differences at different paraxial locations. The central cornea demonstrated a diffuse and regular arrangement of collagen bundles compared with the peripheral or limbal area. The patterns of collagen bundles are denser in the anterior periphery than in the anterior central cornea, but the posterior areas are similar (Fig. 4).

Horizontal Collagen Arrangement Pattern Analysis

In order to quantify the irregularity of the collagen bundle layout, we used image analysis software and developed the simplified pattern analysis method describe above. A representative conversion and analysis are shown in Figure 5. Mean irregularity of central cornea measured at 16.50 ± 0.97 in the superficial layer, 10.92 ± 0.99 in the anterior layer, 6.61 ± 0.52 in the middle layer, and 5.53 ± 0.53 in the posterior layer. Statistical analysis revealed the mean irregularity of each layer to be significantly different from each other. In addition, the irregularity increased when moving from the central cornea to the limbus ($P < 0.001$; Fig. 6).

DISCUSSION

In this study, we used SHG imaging to detail the horizontal collagen arrangement in corneal lamellae as a function of depth and distance from center. The collagen bundles showed complex split-and-merge patterns in the horizontal plane. These split-and-merge patterns were quantified as collagen arrangement irregularity and were found to increase from posterior to superficial lamellae and from the center to the limbus.

Collagen is the main structural component of corneal stroma.¹³ Hence, its collagen architecture determines the

FIGURE 3. Serial tangential second harmonic generation images obtained from the central cornea at different depths (50- μ m steps). (A) Image was taken just below Bowman's membrane (55-year-old white female). Numerous SHG signal-void areas are visible due to the presence of nuclei and oblique fibers attaching to Bowman's membrane. The collagen bundles are smaller in size and the arrangement results in a scattered appearance. The bundles attaching to Bowman's membrane appear as SHG signal-weak areas (*arrowheads*) because of the acute difference in fiber direction. (B–I) The collagen bundle size increases as images approach deeper lamellae. Diffuse, ground-glass appearance (*asterisks*) of the posterior lamellae was observed. *Arrows* indicate corneal folds caused by mild corneal edema. These folds were not observed in (A, B) because of the compact arrangement of collagen in the superficial lamellae. The difference in collagen bundle direction results in the mottled appearance. Images were taken with the epithelium side up at the same magnification and resolution. *Numbers* indicate the depth of image planes.

optical and mechanical properties of the cornea. Abahussin et al.¹⁴ and Meek et al.¹⁵ used x-ray diffraction imaging and reported collagen orientation and distribution in cornea. They found that collagen in the central 8 mm shows marked alignment in the inferior-superior and nasal-temporal directions. In addition, they demonstrated tangentially arranged collagen fibrils circumscribing the central cornea at the limbus (circum-corneal annulus). Although x-ray diffraction is advantageous in quantitative analysis of the bulk orientation of nonfixed corneal collagen fibers, collagen architecture is only extrapolated from the preferential orientation data because x-ray diffraction is unable to produce high-resolution images.

High-resolution imaging of collagen bundles has been previously performed with scanning electron microscopy. In these studies, corneal collagen lamellae crossed at varying angles and split in the anterior-posterior direction as well as in the horizontal direction and heavily interlaced.^{12,15,16} However, the major limitation of electron microscopic study is that fixation and dehydration during processing can alter the normal tissue architecture.

Second harmonic generation signals from corneal collagen (especially type I) is strong enough to obtain images with submicron level resolution. Therefore, corneal collagen architecture can be imaged noninvasively without tissue fixation. Recently, Winkler et al.⁵ used SHG imaging in formalin fixed cornea and found that the anterior-posterior branching points of anterior lamellar corneal fibers were significantly higher than that of middle and posterior lamellar collagen fibers. However, in formalin fixed tissue, the unique advantages of SHG imaging, such as serial optical sectioning in unfixed and near-naturally hydrated tissue are not used. In this study, we obtained serial tangential images in preserved and nonfixed corneas. We believe that minimally altered sample tissues may represent truer architecture of corneal collagen.

The orientation of the collagen fibers with respect to the orientation of laser beam is important when obtaining SHG signals. Fibers oriented parallel to the incoming laser beam generate only forward and not backward scattered signals.^{3,17} Therefore, the mottled appearance in our SHG images is accounted for by the different orientation of collagen fibers. In addition, some areas of mottling are likely due to fibers

FIGURE 4. Collagen arrangement difference is demonstrated between central, peripheral, and limbal cornea at different depths. (A–C) Images were taken from the anterior (A), middle (B), and posterior (C) lamellae of the central cornea (60-year-old white female). The size of collagen bundles appears to increase with increasing depth. (D–F) Images were taken from the anterior (D), middle (E), and posterior (F) lamellae of the temporal peripheral cornea (approximately 4 mm away from the center). Collagen bundles are irregularly scattered in the anterior corneal layers. In the deeper layers, the arrangement of collagen bundles is similar to central cornea. (G–I) Images were taken from the anterior (G), middle (H), and posterior (I) lamellae of the temporal limbal area (approximately 5.5 mm away from the center). Irregular arrangement of collagen bundles is visible throughout the entire depth. Images were taken at the same magnification and resolution. (A–G) Images were taken epithelial side down; (H, I) images were taken endothelial side down.

coursing in and out of the lamellar plane and running in an anterior-posterior direction, parallel to the incoming light beam. Such fibers would likely remain invisible.

In our study, we found that the collagen bundles in the posterior corneal lamellae are more regularly arranged and thicker in width. This finding is consistent with previous reports.^{15,18} It is known that the anterior and posterior lamellae of cornea have different mechanical properties. More compact collagen interwoven randomly in the anterior lamellae might be important for its structural integrity and anterior curvature.³ Muller et al.¹⁹ demonstrated the biomechanical difference between anterior and posterior cornea by using a swelling test. The collagen lamellar density is higher and the arrangement and directionality are more complicated anteriorly than posteriorly. Therefore, the anterior cornea is more resistant to hydrostatic swelling and shear stress.^{15,19,20} In the clinical setting, stromal swelling is usually directed posteriorly and can stress Descemet's membrane into multiple folds.²¹

It is noteworthy that irregularity of the midlimbal cornea was measured to be less than that of the peripheral cornea (Fig.

6C). We postulate that this may be attributable to the relatively poor quality of SHG images taken of the midlimbal layer. At the limbus, laser penetration is relatively poor due to overlapping opaque scleral tissue, which obscure fine collagen bundle arrangement.

There are some limitations to our study. Our simplified pattern analysis method does not fully represent the true collagen bundle pattern of the cornea. During software image analysis, subtle pattern variations may have been missed. Individual accounting of every collagen bundle in an image is a more accurate method. However, limited resolution of images precluded this approach. Another limitation of this study was the presence of stromal folds, especially in the central and posterior corneal layers. These folds are most likely from mild edema during tissue preservation methods and from gravity during imaging. We cannot exclude the possibility that some tissue folds may have affected pattern analysis used in this study. Wu et al.²² reported that corneal edema resulted in distortion of the backscattered SHG signal, which would cause an increase in the 'irregularity' measurement used in this study. In addition, it is possible that all collagen bundles imaged in

FIGURE 5. Horizontal collagen arrangement pattern was analyzed with ImageJ software. (A–C) Corneal stromal pictures (66-year-old white male) were processed by ImageJ with sequential application of bandpass filter and binary conversion. After image processing, the resultant image represented the filtered dark and bright zones of the original image. In processed images, dark areas in original image were converted to white, while the bright areas were converted to black (* & § in [C]). The superficial (A), anterior (B), middle (C), and posterior (D) layers of the central cornea were measured. The crossing density (median cut-off density) was measured at the vertical center of the image (gray area, $30 \times 513 \mu\text{m}$) and each black and white conversion is plotted as a peak in the graph. The number of peaks crossing the median cut-off value (dotted line) of signal intensity is counted and represents the irregularity of collagen bundle arrangement pattern (arrows, [C]).

FIGURE 6. Collagen bundle arrangement irregularity ($n = 7$) was compared between different depths and regions. (A) In the central cornea, irregularity decreased from superficial to posterior lamellae. (B–D) The collagen bundle arrangement became more irregular from the center to the limbus in the anterior (B), middle (C), and posterior (D) cornea. Means and SD are indicated, Kruskal-Wallis test with Bonferroni correction, * $P < 0.001$, § $P = 0.002$, ¶ $P = 0.013$.

square image ($513 \times 513 \mu\text{m}$) do not remain in the same lamellar plane. And some of these out-of-plane collagen bundles might have interfered with accurate analysis of horizontal bundle arrangement.

In summary, SHG imaging reveals unique horizontal collagen bundle arrangements at different depths and locations in the human cornea. The advantage of this technology over previous studies is that the collagen architecture of human cornea was elucidated with minimal tissue alteration. Along these lines, SHG imaging can potentially evolve into a clinically useful diagnostic modality.

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