Multiphoton Fluorescence and Second Harmonic Generation Imaging of the Structural Alterations in Keratoconus Ex Vivo

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PURPOSE. To demonstrate the application of multiphoton fluorescence (MF) and second harmonic generation (SHG) microscopy for ex vivo characterization of the structural alterations of human corneas due to keratoconus.

METHODS. Buttons of keratoconic corneas derived from penetrating keratoplasty were sent for structural analysis with a custom-built multiphoton microscope. Fluorescence detected within the cytoplasm and the SHG signal obtained from collagen were used to demonstrate the morphologic changes in the corneal specimens.

RESULTS. The fluorescent epithelial cells around the apical area were elongated and were aligned parallel to the adjacent collagen fibers. Parallel and centripetal distribution patterns of stromal collagen bundles were demonstrated at different depths within the keratoconic corneas.

CONCLUSIONS. MF and SHG microscopy provides three-dimensional structural analysis of keratoconus ex vivo. It may provide important morphologic information for the investigation of the pathogenesis of keratoconus and may have potential in a clinical setting as an in vivo diagnostic and monitoring system for advancing keratoconus. (*Invest Ophthalmol Vis Sci.* 2006;47:5251–5259) DOI:10.1167/iops.06-0386

Keratoconus is a noninflammatory corneal ectatic disorder characterized by progressive corneal steepening and stromal thinning.¹ Thinning of the corneal stroma, a ruptured Bowman's membrane, and iron deposition in the basal epithelial layers represent the classic histologic signatures of keratoconus.^{2,3} Clinically, this disease may lead to an increase in irregular astigmatism and myopia, causing severe visual distortion.¹

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Several predisposing factors have been proposed for keratoconus, including genetics,⁴ atopy,⁵ eye rubbing,⁶ and the use of contact lens.⁷ Historically, keratoconus has been considered to originate from the corneal epithelium⁸; however, full-thickness involvement of the cornea has been demonstrated in ultrastructural studies.^{3,8–11} Despite histologic and immunohistochemical investigations, the exact pathogenesis of keratoconus remains unknown.

In this study, we used multiphoton microscopy to characterize human keratoconus in ex vivo specimens. The advantages associated with the nonlinear imaging modality using ultrafast, near-infrared light sources include enhanced axial depth discrimination, reduced photodamage, and increased image penetration depth.^{12,13} These properties allow observation of living cells and tissues without inducing detectable damage.^{14–17} Moreover, characteristic fluorescence from cells and extracellular components can help to analyze biological structures of interest.^{18–20} In addition to multiphoton fluorescence (MF) imaging, the nonlinear optical effect of second harmonic generation (SHG) has also been useful for imaging biological structures that lack an inversion symmetry. Biological materials such as collagen, muscle fiber, and microtubules have been shown to be effective second harmonic generators.^{21,22} Therefore, it seems natural to apply SHG imaging to the diseases associated with the cornea which is composed mainly of type I collagen. In particular, since the corneal transparency is closely related to the unique three-dimensional arrangement of stromal collagen fibers,²³ SHG microscopy can be an effective tool for diagnosing abnormalities of the corneal stroma. Previously, we have demonstrated the application of MF and SHG microscopy for imaging the three-dimensional structure of the porcine cornea.²⁴ In our previous work, the MF was effective for visualizing epithelial cells, and the SHG signal derived from collagen was used to image structural motifs within the corneal stroma. In this investigation, we applied multiphoton microscopy to the study of corneal structural alterations due to keratoconus. In this disease, the morphologic changes of the collagenous stroma play an important role during pathogenesis, and a successful demonstration of multiphoton microscopy in characterizing the pathologic features of keratoconus can lead to clinical application of this technique as an effective diagnostic and disease-monitoring tool for this disease.

METHODS

The study protocol was approved by an institutional review board and adhered to the guidelines in the Helsinki Declaration with respect to human subjects in biomedical research.

Specimen Preparation

Three corneal buttons of keratoconus obtained from three patients treated with penetrating keratoplasty were imaged. In addition, one

corneal specimen of pseudophakic bullous keratopathy was imaged for comparison purposes. In all three patients with keratoconus, penetrating keratoplasty was indicated due to the failure of rigid contact lens fitting. The specimens were placed in balanced salt solution and sent for imaging immediately. To prepare the samples for multiphoton examination, the corneal specimens were placed in PBS buffer and covered with a standard coverslip before being mounted on the microscope for imaging.

MF and SHG Microscope

The MF-SHG microscopic system used in this study is similar to the experimental setup previously used for corneal studies.^{24,25} It is a custom-built laser scanning microscopic imaging system based on a commercial upright microscope (model E800; Nikon, Tokyo, Japan). A

FIGURE 1. Keratoconus-I. A 33-year-old woman with bilateral keratoconus underwent penetrating keratoplasty in the left eye, due to failure of visual correction by rigid contact lens fitting. *Top:* The multiphoton cross-sectional imaging of the whole trephined 7.5-mm corneal button. *White arrowheads:* an arcuate, dim demarcating line that may represent the outline of the cone area. *White arrow:* fluorescent mass at the paracentral area proposed to correlate with the apex. In addition, the altered centripetally directed SHG patterns of collagen fibers within the cone area are visualized (*red arrowheads*). *Bottom:* The topography in the patient's left eye before surgery. (The magnified images of areas I and II are shown in Fig. 2.) *Blue:* SHG signal; *green:* fluorescence.

FIGURE 2. Keratoconus-I. The detailed multiphoton images from areas I and II in Figure 1 are shown. Magnified images from selected regions within areas I and II are further analyzed in I-1, I-2, and II-1, II-2. In area I, the region outside the arcuate line, no centripetal pattern of SHG collagen fibers is visible (I-2), and the epithelial cells are round (I-1). However, in area II, the region within the cone area, the parallel pattern of SHG signals that represent collagen structures are identifiable. (II-2), and epithelial cells are shown to be elongated (II-1). *Blue:* SHG signal; *green:* fluorescence.

titanium-sapphire (Ti-sapphire) laser (Tsunami; Spectra Physics, Mountain View, CA) pumped by a diode-pumped solid state laser (Millennia X; Spectra Physics) is used as the excitation source. The 760 nm of the Ti-sapphire laser, capable of inducing fluorescence and SHG from the cornea specimens, is scanned in the focal plane by a galvanometer-driven x-y mirror scanning system (model 6220, Cambridge Technology, Cambridge, MA). The multiphoton images were acquired using water-immersion objectives (XLUMPlanF1, 20×, NA 0.95, Olympus, Tokyo, Japan; Fluor, 40×, NA 0.8, Nikon). To ensure optimal focusing, the laser is beam expanded to ensure overfilling of the objective's back aperture, and a short-pass dichroic mirror (700DCSPXRUV-3p; Chroma Technology, Brattleboro, VT) is used to reflect the incident excitation laser source into the focusing objective. The average laser power at the sample surface is adjusted to ~100 mW, and the MF and SHG signals are collected by the same focusing objective and passed through the dichroic and additional filters before reaching the photodetectors. Although the average power we use is much higher than that used in confocal microscopy (~1 mW), we have observed no photodamage due to one-photon absorption of the near-IR radiation.

FIGURE 3. Keratoconus-II. A 23-year-old woman with bilateral keratoconus underwent penetrating keratoplasty in the left eye due to uncorrectable poor vision. *Top*: the multiphoton cross-sectional image of the entire 7.5-mm trephined corneal button. Clusters of fluorescence are presented in the paracentral area, with a centripetal pattern of SHG collagen fibers surrounding the apical domain (*arrowheads*). The paracentral fluorescent cluster (*arrow*) may correlate with the apical area (*bottom*). Areas I to V are further analyzed in Figure 4 and Table 1. *Blue*: SHG signal; *green*: fluorescence.

Before reaching the detectors, the MF and SHG signals are separated by a secondary dichroic mirror (435DCSX; Chroma Technology). The SHG signal centered at 380 nm was reflected by the secondary dichroic and filtered with a band-pass filter (HQ380/20; Chroma Technology), whereas the longer wavelength fluorescence signal passed through the dichroic mirror and an additional broad-band filter (E435LP; Chroma Technology) before being detected. The detection bandwidths of the MF and SHG signals are 435 to 700 nm and 370 to 390 nm, respectively, and single-photon counting photomultiplier tubes (R7400P; Hamamatsu, Hamamatsu City, Japan) were used to collect the signal photons. For large area scans of the corneal specimens, a two-dimensional stage scanning system (H101; Prior Scientific, Cambridge, UK) was used for specimen translation after each optical scan. The overlapping images acquired in this fashion were assembled into a large-area, high-resolution map of the corneal specimens. For comparison purposes, preoperative topographic images of the keratoconic corneas are also shown.

RESULTS

The large-scale multiphoton scans of the trephined corneal buttons (along with topographic maps) from three keratoconic corneas are shown in Figs. 1, 3, and 5, whereas magnified images of selected regions of interest in the three specimens are shown, respectively, in Figs. 2, 4, and 6. In the multiphoton images, the pseudocolors of blue and green were used to represent the SHG and autofluorescence signals, respectively. Because the corneas were not marked during the surgical removal procedures, the comparison between the multiphoton and topographic images was made by identifying similar features between the two image sets.

In the large area scan of the first keratoconus specimen (keratoconus-I) shown in Figure 1, we observed a significant arcuate demarcating dim line within the SHG-generating corneal stroma (white arrowheads). In addition, the multiphoton image revealed the presence of a fluorescent mass composed of epithelial cells (white arrow). A comparison between the topographic and multiphoton images revealed that the demarcation line and the fluorescent mass observed under multiphoton imaging are consistent in position with the margin of the cone area and keratoconic apex shown in the topographic map. To perform additional analysis, we selected two regions containing epithelial cells in the multiphoton image (I and II). The enlarged images, shown in Figure 2, reveal that both the collagen fiber orientation and the fluorescent epithelial cells of the two areas are morphologically distinct (Fig. 2, I-1 and II-1). First, Figure 2 II-2 shows that the collagen fibers are elongated and surround the fluorescent epithelium, whereas such organization is absent in area I (Fig. 2 I-2). In addition, the fluorescent epithelial cells of region II are elongated and tend to align in the direction of the nearby collagen fibers (Fig. 2 II-1). In contrast, the fluorescent epithelial cells in area I are more circular in appearance (Fig. 2 I-1). These observations support the fact that the elongated collagen and epithelial cells in area

FIGURE 4. Keratoconus-II. Multiphoton images acquired from areas I and III in Figure 3 are further analyzed. In area I, elongated fluorescent epithelial cells are identifiable. In area III, parallel aligned pattern of SHG signals are seen. Magnified images of selected areas within areas I and III are also shown (I-1 and III-1). *Blue*: SHG signal; *green*: fluorescence.

II are due to keratoconus and that most likely, area II corresponds to the apical domain of this specimen.

A similar analysis was performed on the keratoconus-II specimen. A comparison of the topographic and multiphoton images in Figure 3 suggests area I to be near the keratoconic apex. Fluorescent epithelial cells were evident in areas I and IV. In addition, collagen fibers were aligned and surround the proposed apical domain (white arrow). Enlarged images shown in Figure 4 revealed additional features supporting our identification of the keratoconic apex. First, Figure 4 I-1 shows

that the fluorescent epithelial cells are elongated and spindle-like in shape. In addition, Figures 4 III and 4 III-1 reveal the reorganization of the collagen fibers into thick and elongated bundles surrounding the proposed apical domain. These features are consistent with the morphologic features of the collagen fibers and epithelial cells found near the apical domain of the keratoconus-I sample.

In the keratoconus-II specimen, we performed additional analysis of the depth-dependent collagen patterns of five different regions (Fig. 3, areas I-V) and the result is shown in

TABLE 1. Keratoconus II

The depth-dependent SHG images from areas I to V in Figure 3. *Arrow*: the global orientation of SHG collagen bundles. A trend of centripetal orientation can be demonstrated, which becomes increasingly significant at increasing depths.

<div>Area</div> <div>Depth</div>	I	II	III	IV	V
0 μm
100 μm
200 μm
300 μm
400 μm
500 μm
600 μm
700 μm

FIGURE 5. Keratoconus-III. A 27-year-old man with bilateral keratoconus underwent penetrating keratoplasty in the right eye due to uncorrectable vision. *Top:* The multiphoton cross-sectional image of the whole trephined 7.5-mm corneal button at midstroma (depth ~500 μm). The coarse, oblique distribution pattern of SHG signals can be identified. *Bottom:* topography in the right eye before surgery. Selected regions within areas I to V are further analyzed in Figure 6 and Table 2. *Blue:* SHG signal; *green:* fluorescence.

Table 1. In the anterior stroma, the normal interweaving packing of collagen fibers was found. In addition, thick collagen fiber bundles in a centripetal orientation coexisted (Table 1, areas II-V, depth of 100 μm). As the depth increased, the alteration of SHG pattern became more apparent (Table 1, arrow at 400 μm). Instead of the orthogonal-packing collagen pattern normally found in the posterior stroma, the orientation of SHG fibers were markedly altered and became increasingly organized into centripetal patterns surrounding the apex.

In the third and advanced keratoconus sample (keratoconus-III), we acquired a cross-sectional multiphoton image at the midstromal depth of the trephined corneal button (Fig. 5). Comparison with the topographic image suggests that area I corresponds to the apex of this keratoconus specimen. Similar to the previous two cases, SHG microscopy revealed a trend of stromal collagen reorganization toward the paracentral area. This observation is consistent with x-ray diffraction results that revealed the orientation of collagen fibers shift toward an oblique orientation in cases of keratoconus.²⁶ In addition, images of areas I and III in Figure 5 were magnified for further analysis. Figures 6-I and 6-I-1 show fluorescent cells within area

I of the midstroma. These cells are triangular or stellate and are morphologically consistent with activated keratocytes. In addition, parallel alignment of SHG collagen bundles, organized centripetally about the apex, are shown in the detailed images of area III (Figs. 6-III, and 6-III-1). In the keratoconus-III specimen, we also analyzed the detail SHG images of areas I to V at different depths, and the result is shown in Table 2. Similar to findings in the keratoconus-II specimen, centripetally directed patterns of thick collagen bundles were found throughout the cornea.

It should be noted that our observation has revealed thicker corneas in the keratoconus specimens compared with standard samples. This may be because the keratoconus specimens became edematous during the transport and imaging processes, in which buffer solutions were used for sample preservation.

To demonstrate the pathologic differences of keratoconus with other corneal diseases, we prepared and imaged a corneal specimen with pseudophakic bullous keratopathy. The large-area, multiphoton image across the corneal button at the depth of 400 μm is shown in Figure 7. In this image, a relatively homogenous SHG pattern is observed. The centripetal patterns of collagen bundles, evident in the three keratoconus samples, are absent in the edematous sample. This observation is reasonable since the pathologic changes associated with edematous stroma are caused by increased hydration and thickness of corneal stroma. Additional analysis of selected regions was also performed. Enlarged image within areas I and III are shown in Figure 8. In the surface epithelial layer, elongated, spindle-shaped epithelial cells found in keratoconus cases are absent (Fig. 8-I-1, 8-III-1). In addition, the centripetal patterns of thickened SHG collagen bundles are absent in the edematous stroma (Figs. 8-I, 8-I-2, 8-III, 8-III-2). Furthermore, magnified SHG images of areas I-V taken at different depths are shown in Table 3. These images confirm that the centripetally organized collagen pattern found in the keratoconus cases were absent through

FIGURE 6. Keratoconus-III. Detailed multiphoton images from areas I and III in Figure 5 are shown. Activated stellate fluorescent keratocytes are identifiable within the magnified image in area I (I-1), and centripetal and parallel SHG signaling collagen bundles are shown in area III (III-1). *Blue:* SHG signal; *green:* fluorescence.

TABLE 2. Keratoconus III

Depth-dependent SHG images from areas I to V of Figure 5. *Arrows*: the centripetal orientation of SHG signal distribution.

Area Depth	I	II	III	IV	V
0 μm
100 μm
200 μm
300 μm
400 μm
500 μm
600 μm
700 μm
800 μm

the stroma. Instead, the orthogonal packing typical of normal collagen lamellae was found.

DISCUSSION

MF and SHG microscopy has been demonstrated to be a useful technique for normal corneal imaging without the need for additional tissue processing.^{24,27} Nonlinear fluorescence excitation and SHG allow us to image corneal structure with sub-cellular resolution. In this study, we extended our work to demonstrate that the detailed structural alterations of corneal keratoconus can be imaged by multiphoton microscopy, thus demonstrating the potential of this technique in providing structural information underlying keratoconus pathogenesis.

In addition to histologic examinations, other techniques such as confocal microscopy and x-ray diffraction have been applied to the study of the morphologic changes associated with keratoconus.^{26,28–31} Confocal microscopy can demonstrate the morphologic alterations due to keratoconus, particularly at the cellular level. Elongated superficial epithelial cells, similar to our multiphoton results, and hyperreflective nuclei of keratocytes can be visualized. Increased reflectivity and various degrees of haze due to increased background scattering from the stroma have also been demonstrated. Specifically, it was found that the scattering intensity is proportional to the severity of scar. Yet identifying collagen morphology and its involvement is limited with the reflected technique.^{28,29} Therefore, the use of reflected confocal microscopy can provide limited information for exploring keratoconus pathogenesis. However, since the longer, near-IR wavelengths (760 nm in our case) are used for multiphoton excitation, the image resolution achieved using multiphoton microscopy will be inferior compared with that obtained with standard confocal microscopy.

X-ray scattering is another imaging technique that has been applied for demonstrating the corneal structural alterations due to keratoconus.^{26,30,31} Meek et al.²⁶ have demonstrated a significant shift of preferred orientation of collagen, especially

FIGURE 7. Pseudophakic bullous keratopathy. A multiphoton cross-sectional image of the whole trephined 8-mm edematous corneal button at a depth of 400 μm . No significant centripetal SHG collagen bundles are visible. Areas I to V are further analyzed in Figure 8 and Table 3. Blue: SHG signal; green: fluorescence.

FIGURE 8. Pseudophakic bullous keratopathy. The magnified multiphoton images from areas I and III in Figure 7 at the epithelium (I-surface and III-surface) and at a depth of 400 μm were analyzed. No spindle-shaped epithelial cells are identifiable in the magnified images derived from a selected region within areas I and III (I-1 and I-1). The SHG collagen fibers (as shown in area I and III, depth of 400 μm) do not show the centripetal patterns found in the keratoconus specimens. Blue: SHG signal; green: fluorescence.

around the apical area. However, the structural information that x-ray scattering provides is bulk information. Detailed and individualized structural information of individual collagen bundles cannot be obtained. To the best of our knowledge, the present study represents the first noninvasive demonstration of visualizing three-dimensional corneal structural alterations due to keratoconus. Both the cellular and collagenous morphologic alterations in keratoconic corneas can be obtained. Elongation of superficial epithelial cells in response to progressive corneal anterior protrusion can be visualized near the apical area. The

TABLE 3. Pseudophakic Bullous Keratopathy

The depth-dependent SHG images from areas I to V in Figure 7. Unlike the cases of keratoconus, no preferred orientation of SHG signals are found within the edematous cornea.

Area Depth	I	II	III	IV	V
0 μm
100 μm
200 μm
300 μm
400 μm
500 μm
600 μm
700 μm

ability to perform direct and noninvasive imaging of stromal collagen using SHG is one of the major advantages of our approach. We also demonstrated the structural alterations of collagen lamellae with depth-dependent imaging in keratoconus specimens. In addition to the interweaving patterns found in the anterior and posterior regions of normal stroma, patterns of centripetal and thickened collagen bundles directed toward the apical domains were found. The structural alterations of stromal collagen may be the consequence of tissue modulation in the pathologically weakened collagenous stroma in response to intraocular pressure.

Two hypotheses of the pathogenesis of keratoconus have been proposed. First, it was suggested that ectasia is caused by excess tissue degradation or reduced maintenance.³² The second hypothesis suggests that keratoconus is due to the slippage of collagen fiber bundle without tissue loss.³³ These two hypotheses are not mutually exclusive. Tissue degradation may be accompanied by slippage of collagen lamellae, and lamellae slippage caused by the loss of interfibrillar or interlamellar cohesive strength may have resulted from enzymatic degradation. In our work, we demonstrated the alteration of stromal collagen resulting in the generation of centripetally directed

collagen bundles. In the posterior stroma, we found an uneven loss of orthogonal interlacing collagen fibers. Instead, the stromal collagen is organized into elongated and centripetally directed patterns around the apex. This finding may provide confirming evidence for the slippage hypothesis. In this model, the interlacing lamellae is split at the bifurcation point, and the subsequent tissue reorganization results in a parallel pattern of the stromal collagen. However, our result does not contradict the tissue degradation theory, and the sharp demarcation found in involved areas (keratoconus-I) may imply the presence of enzymatic degradation. The apparent activation of quiescent keratocytes (keratoconus-III) indicated by the fluorescence generation associated with increased metabolic activities provide additional evidence of tissue remodeling processes. Similar phenomena are observed within anterior stroma. SHG imaging reveals that the interweaving alignment of anterior stromal collagen, possibly important in maintaining the anterior curvature and structural integrity,^{3,4} has been altered. The involvement of anterior stroma cannot be explained by tissue displacement alone. Instead, enzymatic degradation accompanied by stroma regeneration in response to the intraocular biomechanics will be the more likely explanation for the uneven replacement of the interweaving pattern of collagen fibers by the centripetally directed patterns. The orientation of collagen bundles directed toward the apex implies the corneal biomechanical response in the weakest area, the keratoconic apex. Nevertheless, the detailed processes associated with tissue remodeling due to keratoconus require further investigation.

In conclusion, we demonstrated the use of MF and SHG microscopy for imaging the three-dimensional structural alterations in keratoconus corneal specimens under ex vivo conditions. We believe that this technique can help to provide insights into the pathogenesis of keratoconus and may be developed into an effective clinical diagnostic and monitoring tool for keratoconus.

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