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Second-Harmonic Imaging Microscopy of Normal Human and Keratoconus Cornea

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Abstract

PURPOSE—The purpose of this study was to evaluate the ability of second-harmonic imaging to identify differences in corneal stromal collagen organization between normal human and keratoconus corneas.

METHODS—Six normal corneas from eye bank donors and 13 corneas of patients with keratoconus, obtained after penetrating keratoplasty were examined. A femtosecond titanium-sapphire laser with 800-nm output was used to generate second-harmonic signals collected at 400 nm from central and paracentral corneal tissue blocks. Three-dimensional (3-D) data sets were collected and reconstructed to evaluate the location and orientation of stromal collagen lamellae.

RESULTS—Imaging of second-harmonic signals combined with 3-D reconstruction of the normal cornea identified a high degree of lamellar interweaving, particularly in the anterior cornea. Of note was the detection of lamellae that inserted into Bowman's layer and were oriented transverse to the corneal surface, penetrating posteriorly approximately 120 μ m. In keratoconus corneas, imaging second-harmonic signals identified less lamellar interweaving and a marked reduction or loss of lamellae inserting into Bowman's layer in 12 of 13 cases, particularly in regions associated with cone development without breaks in Bowman's layer or scarring.

CONCLUSIONS—Compared with normal adult corneas, marked abnormalities were detected in the organization of the anterior corneal collagen lamellae of keratoconus corneas by second harmonic imaging. These structural abnormalities are consistent with the known changes in collagen organization and biomechanical strength of keratoconus.

Recent studies have reported on the use of femtosecond lasers to generate second-harmonic signals from collagen to probe corneal collagen organization at high spatial resolution and contrast.^{1–4} Additional studies indicate that SHG signals can be obtained throughout the cornea in normal eyes without the need to section and process tissue and that data can be used to study differences in the collagen organization between the cornea and sclera,⁵ assess femtosecond laser photodisruption of collagen fibers,⁶ study thermally induced changes in collagen structure,⁷ and determine gross pathologic changes and disorganization due to specific gene defects in transgenic mice.⁸

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Since the biomechanical strength of the cornea is generally thought to be associated with the axial strength of the collagen fibrils, understanding the 3-D collagen organization is important to understanding the mechanisms controlling corneal shape and biomechanics. In theory, 3-D imaging of second-harmonic signals could be useful as a noninvasive, in vivo imaging approach to evaluate collagen orientation and hence biomechanical strength in patients. However, little is known regarding the ability of imaging second-harmonic signals to detect differences in collagen organization in various corneal disorders, nor how potential differences relate to biomechanical strength.

In this study, we evaluated the ability of imaging second-harmonic signals to detect differences between the normal corneal lamellar organization and keratoconus, which is an acquired or genetic disorder that has altered biomechanical properties leading to progressive thinning and steepening of the paracentral cornea.⁹ Earlier studies using transmission electron microscopy and x-ray diffraction have shown an alteration in the anterior corneal lamellar organization of the keratoconus cornea compared with normal consisting of reduced lamellar interweaving.^{10,11} In this report, we demonstrate that imaging of second-harmonic signals can be used to detect highly interwoven lamellar organization of the normal adult anterior corneal stroma as well as a prominent population of lamellae that insert into Bowman's layer and extend posteriorly approximately 120 μm . In contrast, keratoconus corneas showed a marked decrease in lamellar interweaving and an absence or loss of lamellae that inserted into Bowman's layer. These findings suggest that development of a clinical approach to obtaining second-harmonic signals from patients may be useful in the diagnosis and evaluation of keratoconus and other corneal disorders.

Methods

Patients

This clinical study was approved by the Institutional Review Board of the University of California Irvine (UCI) and Yamaguchi University Hospital. All human specimens were treated according to the World Medical Association's Declaration of Helsinki. Normal corneas not suitable for corneal transplantation due to age or abnormal serology but otherwise normal were obtained from the San Diego Eye Bank. Normal corneas arrived overnight in preservation media and were inspected by biomicroscopy, to ensure the absence of any corneal disease. Corneas were then fixed in 2% paraformaldehyde in phosphate-buffered saline (pH 7.4). Keratoconus corneas were requested from two institutions: the University of California Medical Center and Yamaguchi University Hospital. Sequential penetrating keratoplasty buttons from patients who had a diagnosis of keratoconus were obtained fresh and placed whole in fixative before shipping to the laboratory. One institution required submission of tissue to pathology for confirmation of diagnosis (UCI), thus limiting the amount of sample available for examination to a half corneal button.

Preparation of Tissue

Corneal tissue was dissected into smaller tissue blocks (1–1.5-mm blocks) taken from the central and paracentral regions, washed in PBS, mounted on glass coverslips with 50% glycerol/PBS, and then imaged. Selected samples were further stained overnight at 4°C with phalloidin (Alexa Fluor 488 phalloidin; Invitrogen-Molecular Probes, Eugene, OR) and 5 μM of red nucleic acid stain (SYTO red; Invitrogen-Molecular Probes, Eugene, OR) in 50% TD buffer (0.5% dimethyl sulfoxide [DMSO], 0.5% Triton X-100, and 2.5% dextran 40 in PBS [pH 7.4]) to identify actin cytoskeleton and nuclei, respectively.

Conventional and Multiphoton Confocal Microscopy

Specimens were placed on a microscope (Axiovert 200; Carl Zeiss Meditec, Inc., Jena, Germany) and imaged with a 40 $\times$ (NA, 1.3) oil-immersion objective lens (Carl Zeiss Meditec, Inc.) at a 190- μ m working distance. The fluorescent signal detection from phalloidin and the red stain were obtained by using the 488- and 633-nm laser lines of the argon and red helium-neon lasers, respectively. Emitted light was detected using 500- to 550-nm and 650-nm long-pass filters for phalloidin and the nucleic acid stain, respectively. Two-photon second-harmonic signals from collagen were generated with a mode-locked titanium:sapphire laser (Chameleon; Coherent Inc., Santa Clara, CA). We previously reported that the optimum wavelength to generate second-harmonic signals from human corneal collagen was 800 nm.¹² Laser light was circularly polarized with a $\frac{1}{4}$ waveplate interposed between the laser and microscope. Forward scattered or transmitted signals that passed through the tissue were collected with an 0.8 NA condenser lens with a narrow band-pass filter (400/50) placed in front of the transmission light detector. Backward-scattered signals were collected by the microscope objective and detected over the wavelengths from 377 to 430 nm with the detector on microscope (META detector on the model 510; Carl Zeiss Meditec, GmbH). With the multitrack mode of the equipment, we obtained sequential, en face second-harmonic and single-photon fluorescent signals from the same optical slice. All samples were scanned using a 1- μ m z-axis step size to generate 3-D data sets extending from the corneal epithelial surface through Bowman's layer and into the anterior stroma to a depth of 150 μ m. All images were recorded as 12-bit, 512 $\times$ 512 images. 3-D data sets were then reconstructed (LSM Image Examiner; Carl Zeiss Meditec, GmbH). For each corneal sample, a minimum of three 3-D data sets were collected from each corneal block. To determine whether Bowman's layer was intact or broken, additional corneal blocks were prepared and scanned.

Results

Patients

Six normal human corneas (three male and three female, mean age, 63.8 $\pm$ 22.5 years; range, 36 – 84) were obtained. Because younger aged corneas are needed for donor material, the normal corneal samples tended to come from older individuals, although two of the samples were representative of the mean age of the keratoconus samples. Thirteen keratoconus corneal buttons from 10 males and 3 females (mean age, 39.3 $\pm$ 14.7 years; range, 19 – 60) were obtained sequentially after penetrating keratoplasty for keratoconus from the two different institutions. Four buttons were whole, while in the remaining samples, half of the button had been submitted to pathology as required by the hospital (UCI) to confirm the diagnosis.

Second-Harmonic Signals from Normal Corneas

As mentioned in the Methods section, sequential images taken parallel to the surface of the cornea were obtained through the anterior corneal stroma to generate 3-D data sets. Representative forward and backward second-harmonic images are shown in Figure 1. At the level of Bowman's layer, the forward detector showed a distinct punctate signal (Fig. 1A), whereas the backward detector showed a more indistinct and diffuse signal (Fig. 1B). Below Bowman's layer, 10 μ m deeper from the plane shown in Figure 1A, the forward detector showed narrow bands of short collagen fibers aligned in random orientations (Fig. 1C) that were consistent with the narrower and thinner lamellae comprising the anterior corneal stroma that have been identified by TEM and SEM.^{13,14} Although the image was less distinct, the backward detector showed a clearly interwoven pattern of collagen lamellae at the same focal plane (Fig. 1D). At a plane 50 μ m below Bowman's layer, the forward detector showed collagen bands organized into longer and wider lamellae that remained oriented in random directions (Fig. 1E), whereas the backward detector showed a similar widening of the lamellae (Fig. 1F). A z-series of images collected by the forward detector were then three-dimensionally

reconstructed and rotated along the y-axis to generate an x, z projection. These datasets showed that many of the short lamellar bands represented much longer lamellae that ran transverse to the surface of the cornea, penetrating deeper into the anterior cornea (Fig. 1G). These lamellae appeared to originate at Bowman's layer (asterisk) and extend transversely at an average angle of $23 \pm 8^\circ$ (range, $9 - 43^\circ$; $n = 45$), to a depth of $>120 \mu\text{m}$. Rotation of the 3-D projection showed that these lamellae were highly interwoven (data not shown). Since the backward detected images were less distinct, a $10 \times 230\text{-}\mu\text{m}$ slice through the 3-D data set was reconstructed and rotated along the y-axis to show a cross section through the stroma (Fig. 1H). These cross-sectional images showed a distinct layer of collagen having a diffuse or indistinct organization consistent with Bowman's layer (Fig. 1H, asterisk). Below Bowman's layer multiple lamellae of various sizes and orientations running both parallel and transverse to the corneal surface could be detected. This organizational pattern appeared remarkably similar to that of routine hematoxylin and eosin-stained corneal tissue sections.

Representative maximum intensity projections that colocalized forward and backward detected collagen signals from normal corneas are shown in Figure 2 for all six control subjects. In all control subjects, prominent lamellae appearing to insert into Bowman's layer were detected throughout the anterior cornea. In comparing corneas of different ages, no differences were noted between younger (Figs. 2A, 2B, ages 36 and 38 years) and older (Figs. 2E, 2F, both aged 84) corneas.

Keratoconus Corneas: Regions Underlying Intact Bowman's Layer

Initially, the nonscarred regions of the cornea were evaluated for lamellar organization. Of the 13 cases evaluated, five buttons showed no evidence of scarring or breaks in Bowman's layer, whereas the remaining eight cases had detectible breaks with regions of scarring. In a representative subject (32-year-old male) with corneal scarring, second-harmonic signals taken by the forward detector (Figs. 3A, 3C, 3E, 3G) and backward detector (Figs. 3B, 3D, 3F, 3H) are shown in a region where Bowman's layer was intact (Figs. 3A, 3B). In this representative case, the cornea underlying intact Bowman's layer showed a distinct decrease in the number of short lamellae detected by the forward detector (Fig. 3C) and less lamellar interweaving was detected by the backward detector (Fig. 3D) compared to normal. Deeper into the anterior stroma ($50 \mu\text{m}$), the loss of lamellae detected by the forward detector was more dramatic (Fig. 3E), and lamellar interweaving was completely absent in the backward detector image (Fig. 3F). 3-D reconstruction of the images obtained from the forward detector confirmed the single-image data and showed that the number of lamellae inserting into Bowman's layer (asterisk) was remarkably reduced in some regions (Fig. 3G, arrow) and when present only extended a short distance into the anterior cornea. Reconstruction of a $10 \times 230\text{-}\mu\text{m}$ slice through the 3-D data set taken from the backward detector also confirmed the marked lack of lamellar interweaving below Bowman's layer (asterisk), showing predominantly a lamellar alignment parallel to the corneal surface (Fig. 3H), which was distinctly different from that of normal adult cornea (see Fig. 1H).

Representative maximum-intensity projections that colocalized forward and backward detected collagen signals from regions of cornea where Bowman's layer was intact without corneal scarring are shown in Figure 4 for all 13 cases (Cases are arranged in order of age; Fig. 4A, 19 years; 4M, 60 years). Of these 13 cases, 5 buttons showed no evidence of breaks in Bowman's layer or corneal scarring in any region evaluated (Figs. 4B, 4H, 4I, 4J, 4M; 4E, same patient as shown in Fig. 3). Overall, in 12 of 13 cases, there was a wide range of changes in lamellar organization with at least two cases having almost complete loss of lamellae inserting into Bowman's layer (Fig. 4B, 4F). Of note, one of the two cases (Fig. 4B) was from a whole corneal button that showed no evidence of breaks in Bowman's layer or corneal scarring. The remaining cases showed regions where lamellae did not insert into Bowman's

layer (asterisk; Figs. 4A, 4E, 4I, 4J, 4M), severe disruption (Fig. 4G, 4L), and lamellar thinning (Fig. 4D) or shortening (Fig. 4C, 4H). One case (Fig. 4K) appeared essentially normal, but data were obtained from only one half of the corneal button, the other half having been submitted to pathology. In comparing these changes to similar-aged, normal corneas, note that the cases in Figures 4E–M have an age range (32–60 years) similar to the first three normal corneas presented in Figure 2 (36–60 years) and yet show a markedly different organizational pattern.

Keratoconus Corneas: Regions with Absent or Discontinuous Bowman's Layer

In regions where there were breaks in Bowman's layer, a strong signal was detected by the forward detector (Fig. 5A, arrow; same patients as shown in Fig. 3) in the same optical plane where the backward detector showed an indistinct and diffuse signal (Fig. 5B). Below Bowman's layer (10 μm) no lamellae inserting into Bowman's layer were detected by the forward detector (Fig. 5C) or lamellar interweaving by the backward detector (Fig. 5D). A similar pattern was detected deeper within the cornea (Figs. 5E, 5F). 3-D reconstruction of the forward-detected images showed marked deposition of collagen within the break at Bowman's layer (Fig. 5G, arrow) suggesting fibrosis, but no transverse lamellae or lamellar interweaving was detected by either the forward or backscattered detector (Fig. 5H, arrow).

Representative maximum intensity projections colocalizing the forward and backward signals in the eight keratoconus corneas with absent or discontinuous Bowman's layer were generated to evaluate further the changes in the collagen organization and presence of lamellae inserting into Bowman's layer (Fig. 6). In these eight cases, five showed clear breaks in Bowman's layer (BL) with fibrotic tissue deposited within the breaks (between arrows; Figs. 6A, 6B, 6F–H), which in one case formed significant scar tissue between the corneal epithelium and Bowman's layer (Fig. 6B, asterisk). In the remaining three corneas, there was complete loss of detectible Bowman's layer (Figs. 6C–E). In seven of the eight cases, there was severe disruption of lamellae that inserted into Bowman's layer, which for the most part were completely absent. In the remaining case (Fig. 6H), lamellae inserting into Bowman's layer appeared limited to the region outside the break in Bowman's layer (Fig. 6H, arrowhead).

The relationship between breaks in Bowman's layer and corneal fibrosis was further investigated by counterstaining with phalloidin and nucleic acid stain (SYTO red; Invitrogen-Molecular Probes) to identify cell borders and nuclei respectively (Fig. 7). In a corneal button obtained from a 26-year-old male keratoconus patient, the break in Bowman's layer was detected in an x, z projection through the 3-D data set (Fig. 7A, between arrows) showing actin (green), nuclei (red), forward (cyan), and backward (magenta) detected collagen. Fibroblastic cells (arrowheads) were detected on the epithelial side of Bowman's layer away from the region of scarring between the broken ends of Bowman's layer (asterisk). Of interest, a gap was detected between Bowman's layer and the overlying epithelium (Fig. 7A, double-headed arrow) that did not appear to show a detectible collagen signal; note the absence of a cyan or magenta signal in this region. While this gap could be an artifact, similar gaps were detected in all keratoconus buttons that showed breaks in Bowman's layer and were counter-stained with phalloidin and nucleic acid red stain (data not shown). It is likely that this gap contains abnormally deposited extracellular matrix material such as type VII collagen, perlecan, or other basement membrane components that have been identified in this region by immunocytochemistry.¹⁵ In x, y planes through the region of the gap (Fig. 7B), fibrotic tissue and fibroblasts appeared to extend from the break in Bowman's layer. Collagen was not detected in the adjacent region, though cells clearly extended beyond the area of fibrosis. In x, y planes through Bowman's layer, an enhanced forward detected signal showing a Y-shaped deposit of collagen within Bowman's layer (BL) that was oriented directly across the break (Fig. 7C, arrowheads) was noted. Increased scattering along the break was also noted by the

backward detector, showing collagen deposited transverse rather than parallel to the break (Fig. 7D).

Discussion

In this study, noninvasive imaging of second-harmonic– generated signals was used to visualize the structural lamellar organization of the anterior cornea. This imaging approach identified a high degree of lamellar interweaving in the anterior cornea with a distinct population of lamellae appearing to insert into Bowman's layer and run transverse to the corneal surface to a depth of approximately 120 μm . In a recent report, these lamellae that are identified by second-harmonic imaging have been shown to be distinct to the human and not observed in mouse or rabbit corneas.¹² Of note, the transverse orientation with insertion into Bowman's layer suggests that the lamellae have an important mechanical function. In dogfish, "sutural" lamellae that insert into Bowman's layer and Descemet's membrane are thought to be responsible for the maintenance of corneal deturgescence in the absence of a functioning corneal endothelium.^{16,17} In the human cornea, such sutural lamellae may play a similar role and provide rigidity to the anterior corneal stroma as well as define the corneal shape and curvature.

More important, second harmonic imaging was able to detect distinct differences in the organizational pattern of lamellae in 12 of 13 keratoconus samples including a marked loss or decrease in anterior lamellar interweaving and lamellae that inserted into Bowman's layers. The remaining case, which did not show loss of sutural lamellae or anterior lamellar interweaving, was from a keratoconus patient from which only half of the corneal button was available for analysis, the remaining half having been submitted to pathology. These findings provided by a noninvasive imaging technique are consistent with previous light and electron microscopic studies of keratoconus corneas showing uniform, orthogonally arranged lamellar layers in the anterior stroma, with little or no lamellar interweaving.¹⁰ In addition, studies using x-ray scattering have demonstrated that keratoconus corneas show displacement of collagen orientation suggestive of lamellar slippage, which for anterior lamellae may involve disinsertion of lamellae from Bowman's layer.¹¹ Overall, these findings suggest that the mechanical weakness of keratoconus corneas that lead to corneal thinning and cone formation may be associated with the loss of a distinct population of anterior stromal sutural lamellae that insert into Bowman's layer and anchor deeper within the stroma. Clearly, future development of second-harmonic imaging may provide a clinical tool to assess further this mechanism as well as help in diagnosing and observing the progression of this disorder.

Since this study was performed on pathologic specimens obtained after penetrating keratoplasty, the importance of these findings remains unclear. Although it is possible that the changes identified are associated with the end-stage disease, the finding that there was marked loss of lamellae inserting into Bowman's layer in at least one whole corneal button showing no breaks in Bowman's layer or corneal scarring suggests that the loss of these lamellae may precede end-stage disease. In support of this conclusion, a recent 3-D SEM study of keratoconus showed sharply edged defects in Bowman's layer.¹⁸ These defects may be associated with the loss of lamellae that directly insert into the edge of Bowman's layer. Although these investigators have suggested that the changes in Bowman's layer may be the first signs of keratoconus,¹⁸ in our study, four patients without any evidence of breaks in Bowman's layer exhibited marked changes in the number of lamellae that inserted into Bowman's layer, presumably occurring in conjunction with paracentral corneal thinning and steepening. We therefore suggest that the changes observed in these structures may be responsible in part for the changes in the mechanical properties of keratoconus corneas that lead directly to altered corneal shape. Such a hypothesis is consistent and supportive of the hypothesis proposed by Bron¹⁹ and Muller et al.²⁰ that anterior lamellar interweaving controls corneal shape.

The loss of lamellae inserting into Bowman's layer may also explain in part the findings by x-ray scattering that the preferred horizontal and vertical orthogonal orientation of stromal collagen is altered in keratoconus.^{11,21} In the more recent report, scattering data indicate that collagen orientation in keratoconus assumes a more curved or tangential alignment to the cone, suggesting slippage of lamellae during progression of disease.¹¹ This finding supports a long-postulated alternative view of the pathogenesis of keratoconus involving slippage with no degradation of tissue,²² as well as the more recent observations that surface topographic changes in keratoconus suggest that the disease is an extreme form of corneal warpage rather than ectasia or corneal stretching perhaps caused by lack of structural integrity brought on by stromal degeneration and external forces.²³ Since insertion of lamellae into Bowman's layer and extensive interweaving of these lamellae deeper within the anterior cornea may greatly increase the mechanical strength of the anterior cornea, the selective loss of these structures may markedly reduce the interlamellar cohesion and facilitate slippage of lamellae. Indeed, Meek et al.¹¹ have suggested such a mechanism of slippage in the anterior cornea with disinsertion of lamellae from Bowman's layer in a recent report.

Although the data may suggest degradation of lamellae that insert into Bowman's layer, it should be noted that in keratoconus corneas, total collagen content is generally not substantially altered. Because these lamellae most likely represent a minor population of the overall collagen lamellae, it is not likely that their selective removal or loss would be detected in bulk collagen assays. Furthermore, it is possible that these lamellae may be replaced by orthogonally arranged collagen lamellae running parallel to the corneal surface with no overall decrease in collagen content.

Several questions remain concerning the disappearance of lamellae that insert into Bowman's layer. Since in this study we did not have age-matched control eyes, we do not know if these structures develop in older eyes or are developmentally regulated. In support of a developmental regulatory program is the finding that younger normal corneas aged 36 and 38 years showed a similar distribution of lamellae inserting into Bowman's layer to those of older individuals, 60 to 84 years. Therefore, at least after adolescence, there does not appear to be any changes in the organization of these structures. However, further study is necessary to address this question. Second, the inability to detect these structures in keratoconus does not necessarily mean that these structures have been lost. It is possible that changes in the collagen organization or binding of other extracellular matrix protein and glycoprotein may block the ability of the collagen to generate second-harmonic signals. However, it should be noted that in the *x*, *z* projections from the backscattered signal, the organization of the collagen appeared predominantly orthogonal (Fig. 3H) in contrast to the interwoven pattern seen in normal (compare with Fig. 1H). Further, there were no void regions in the reconstructions from the keratoconus corneas that suggested the presence of lamellae inserting into Bowman's layer and extending deeper within the cornea. Taken together, these findings suggest that the loss of second-harmonic signals is related to the disappearance or absence of these structures.

Certainly, additional study is warranted to evaluate the lack of these structures in keratoconus corneas. Imaging of second-harmonic signals is a noninvasive procedure that theoretically can be used in patients, at least to detect backscattered SHG signals. Further development of this technology may provide important clues to the pathogenesis of keratoconus, as well as the detection of early or suspect keratoconus and the structural organization of the cornea in normal individuals. In addition, identifying the contribution of sutural lamellae to the corneal biomechanical strength may be important to understanding how collagen organization controls corneal shape, which may lead to better insights into the basic mechanisms underlying refractive errors such as myopia, hyperopia, and astigmatism, and may help to understand the effects of refractive surgery and the potential risk of corneal ectasia after LASIK.

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Figure 1.

Second-harmonic signals from normal adult cornea detected with the forward (**A, C, E, G**) and backward (**B, D, F, H**) detector. (**A, B**) Bowman's layer; (**C, D**) 10 μm below (**A**); (**E, F**) 50 μm below (**A**). (**G**) 3-D reconstruction of the data set from forward detector showing a maximum intensity projection rotated 90° in the y-axis through 230 μm of anterior stroma. (**H, ***) Bowman's layer. Bar, 50 μm .

Figure 2. 3-D reconstruction of data sets for all control, normal corneas from the forward and backward detectors showing maximum intensity projections rotated 90° in the y-axis. (A–F) Images from donors aged 36, 38, 60, 81, 84, and 84 years, respectively. Bar, 50 μm .

Figure 3.

Second-harmonic signals from keratoconus corneas detected with the forward (**A, C, E, G**) and backward (**B, D, F, H**) detector. (**A, B**) Bowman's layer; (**C, D**) 10 μm below (**A**); (**E, F**) 50 μm below (**A**). (**G**) 3-D reconstruction of the data set from forward detector showing a maximum intensity projection rotated 90° in the y-axis through 230 μm of anterior stroma. Note that lamellae inserting into Bowman's layer are absent (*arrow*) or shortened. (**H**) 3-D reconstruction of a 10- μm -thick slice through the data set from the backward detector showing a maximum intensity projection rotated 90° in the y-axis through 10 μm of anterior stroma. (*) Bowman's layer. Bar, 50 μm .

Figure 4.

3-D reconstruction of data sets for all 13 cases of keratoconus from the forward and backward detectors showing maximum intensity projections rotated 90° in the y-axis. (A–M) Images from donors aged 19, 22, 23, 26, 32, 33, 34, 48, 49, 52, 56, 57, and 60 years, respectively. (*) Regions of disrupted lamellae inserting into Bowman's layer. Bar, 50 μ m.

Figure 5.

Second-harmonic signals from keratoconus cornea detected with the forward (**A, C, E, G**) and backward (**B, D, F, H**) detector in a region showing a break in Bowman's layer. (**A, B**) Bowman's layer; *arrow*: break with increased collagen deposition; (**C, D**) 10 μm below (**A**); (**E, F**) 50 μm below (**A**); (**G**) 3-D reconstruction of the data set from forward detector showing a maximum intensity projection rotated 90° in the y-axis through 230 μm of anterior stroma. Note absence of lamellae that insert into Bowman's layer and increased collagen deposition at the break in Bowman's layer (*arrow*). (**H**) 3-D reconstruction of a 10 $\times$ 230- μm slice through the data set from the backward detector showing a maximum intensity projection rotated 90° in the y-axis through a 10- μm cross section. Note absence of lamellar interweaving and disruption of lamellar organization at break (*arrow*). (*) Bowman's layer. Bar, 50 μm .

Figure 6.

3-D reconstruction of data sets showing forward and backward detected images of the eight cases of keratoconus with breaks in Bowman's layer (between *arrows*: **A**, **B**, **F–H**). Note the fibrosis above Bowman's layer (**B**, *) and the absence of lamellae that insert into Bowman's layer in all samples except (**H**), which shows loss of these lamellae underlying the break in Bowman's layer (*arrowhead*). Epi, epithelium; BL, Bowman's layer; Str, stroma. Bar, 50 μ m.

Figure 7.

Spatial relation between broken Bowman's layer and corneal stromal cells showing forward (cyan) and backward (magenta) detected collagen, nuclei (red), and actin (green). (A) *x*-, *z*-Slice through the 3-D data set at the break in Bowman's membrane (between arrows) showing collagen deposited at the break (*) with fibroblasts extending along the epithelial side of Bowman's layer (arrowheads) and a gap between Bowman's layer and the corneal epithelium (double-headed arrow). (B) *x*-, *y*-Plane showing fibroblasts appearing to migrate from fibrotic collagen deposited above Bowman's layer (cells are same as those indicated in C at arrowheads). (C) *x*, *y*-Plane showing forward detected collagen signal at level of Bowman's layer (indicated as BL). Note the deposition of collagen transverse to the break (arrowheads). (D) *x*, *y*-Plane showing backward detected collagen signal. Note collagen deposited along the break. Bar, 50 μ m.