Nonlinear Optical Macroscopic Assessment of 3-D Corneal Collagen Organization and Axial Biomechanics

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PURPOSE. To characterize and quantify the collagen fiber (lamellar) organization of human corneas in three dimensions by using nonlinear optical high-resolution macroscopy (NLO-HRM) and to correlate these findings with mechanical data obtained by indentation testing of corneal flaps.

METHODS. Twelve corneas from 10 donors were studied. Vibratome sections, 200 μm thick, from five donor eyes were cut along the vertical meridian from limbus to limbus (arc length, 12 mm). Backscattered second harmonic-generated (SHG) NLO signals from these sections were collected as a series of overlapping 3-D images, which were concatenated to form a single 3-D mosaic (pixel resolution: 0.44 μm lateral, 2 μm axial). Collagen fiber intertwining was quantified by determining branching point density as a function of stromal depth. Mechanical testing was performed on corneal flaps from seven additional eyes. Corneas were cut into three layers (anterior, middle, and posterior) using a femtosecond surgical laser system and underwent indentation testing to determine the elastic modulus for each layer.

RESULTS. The 3-D reconstructions revealed complex collagen fiber branching patterns in the anterior cornea, with fibers extending from the anterior limiting lamina (ALL, Bowman's layer), intertwining with deeper fibers and reinserting back to the ALL, forming bow spring-like structures. Measured branching-point density was four times higher in the anterior third of the cornea than in the posterior third and decreased logarithmically with increasing distance from the ALL. Indentation testing showed an eightfold increase in elastic modulus in the anterior stroma.

CONCLUSIONS. The axial gradient in lamellar intertwining appears to be associated with an axial gradient in the effective elastic modulus of the cornea, suggesting that collagen fiber intertwining and formation of bow spring-like structures provide structural support similar to cross-beams in bridges and

large-scale structures. Future studies are necessary to determine the role of radial and axial structural-mechanical heterogeneity in controlling corneal shape and in the development of keratoconus, astigmatism, and other refractive errors. (*Invest Ophthalmol Vis Sci.* 2011;52:8818–8827) DOI:10.1167/iov.11-8070

The human cornea fulfills a dual role, acting both as a physical barrier to maintain ocular integrity and as the primary refractive element responsible for transmitting and focusing light onto the retina. The mechanical strength and shape of the cornea are largely dependent on the corneal stroma, which makes up over 90% of corneal thickness. The major structural element comprising the corneal stroma is collagen, which accounts for approximately 70% of the corneal total dry weight.¹

Collagen assembles to form long fibrils that in the human cornea show a uniform diameter of approximately 31 to 34 nm.² Fibrillar size, spacing, and stability are regulated by non-fibrillar collagen and proteoglycans, found in the interfibrillar matrix.³ Collagen fibrils are generally organized into independent bundles or fibers, which in the cornea have been referred to as lamellae. Collagen fibers are approximately 1 to 2 μm thick and 10 to 200 μm wide and are thought to traverse the entire cornea from limbus to limbus. The biomechanical properties of the cornea are thought to be controlled by several factors, including collagen fiber organization of the cornea.⁴ Collagen organization hence influences corneal shape, which plays an important role in determining visual acuity.

The mechanisms that control corneal shape remain poorly understood, and the spatial organization of corneal collagen has been the focus of a large body of research using a variety of imaging modalities. The corneal nanostructure has been studied using electron microscopy,⁵ which has the capability of resolving individual collagen fibrils. X-ray diffraction has also been used to analyze bulk collagen alignment across the entire cornea.^{6,7} The combined findings of these studies have led to a comprehensive model of collagen distribution which has been the basis of our understanding of corneal biomechanics. In this model, broad collagen fibers run in-plane across the width of the cornea and are stacked vertically in approximately 200 separate planes. Lamellae within each plane are preferentially aligned along the horizontal or vertical meridians of the cornea,⁷ and each layer has a preferential alignment vector that is rotated approximately 90° relative to its neighbors. Peripherally, fibers show a more tangential disposition, forming a circular pattern that follows the circumference of the cornea.⁶ In conjunction with collagen inserting from the limbus, these patterns are thought to determine the shape of the cornea. In general, collagen fibers are thought to be separate entities, with their interaction limited to interweaving with adjacent lamellae.¹ Overall, current computer models of human corneas are generally based on this model of corneal collagen organization.⁸

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Development of nonlinear optical (NLO) imaging techniques such as second harmonic-generated (SHG) imaging has emerged as a powerful new tool for investigating collagen organization.⁹ SHG is a nonlinear, absorption-free optical process created in tissue illuminated with femtosecond-range infrared laser pulses. Briefly, during SHG the extremely high field strengths associated with ultrashort laser pulses cause an oscillating polarization in certain molecules, such as collagen, that results in the emission of light at exactly half the wavelength of the laser beam. Because of the nature of the process, it occurs only in the focal plane of the laser beam, yielding high lateral and axial resolution. Furthermore, SHG is limited to structures that lack central symmetry, making it a highly specific imaging paradigm that does not require staining. Finally, because of the high axial resolution and the increased penetration depth of infrared light, it allows for 3-D image acquisition through the full thickness of the cornea.

Using this imaging paradigm, SHG studies showed that collagen is arranged in a more complex fashion than previously thought. Rather than forming highly parallel, distinct fibers that run largely uninterrupted, collagen appears to be arranged in fibers that can change direction and interact with adjacent fibers in ways beyond mere interweaving, such as branching and fusing. SHG studies have also revealed the presence of sutural fibers that run upward and insert into Bowman's layer.¹⁰ These fibers have been found only in the anterior portion of the cornea, indicating that the collagen organization in the anterior cornea is more complex and thus pointing toward an axial heterogeneity in collagen fiber interconnectivity. Interestingly, keratoconus corneas are marked by an absence of sutural fibers, which suggests that fiber interconnectivity influences rigidity and possibly shape.^{11,12}

Recently, SHG imaging has been used to reconstruct the organization of collagen in the corneal stroma using nonlinear optical high-resolution macroscopy (NLO-HRM).¹³ This technique combines SHG imaging with automated image acquisition and concatenation procedures to generate large-scale, 3-D image mosaics several millimeters across that retain extremely high lateral and axial resolution. The resulting data qualitatively show that collagen fibers, particularly in the anterior stroma, tend to branch into multiple distinct fibers that then fuse with each other or even with unrelated fibers originating from a different layer. This effect causes an intertwining of fibers rather than an interwoven pattern where fibers remain separate entities.¹⁴ Based on these observations, we hypothesize that intertwining creates structural links between multiple fibers and leads to increased mechanical rigidity, predominantly in the anterior cornea.

MATERIALS AND METHODS

Tissues

Eyes of 10 donors (12 total eyes) were obtained from the San Diego Eye Bank after institutional board approval and in accordance with the Declaration of Helsinki. The globes were inspected under a stereomicroscope to qualitatively assess the state of the cornea with regard to swelling and transparency and to ensure the absence of any major visible defects or abnormalities. Donor and eye history for all 12 eyes and the respective analysis are presented in Table 1.

HRMac Analysis

Sample Preparation. A total of five eyes from five different donors aged 34 to 67 years (mean, 57.2 ± 13.5 years) were used for analysis of collagen fiber organization by HRMac. Central corneal thickness on receipt was in excess of 800 μm because of postmortem swelling. The corneas were initially thinned by infusing phosphate-buffered saline (PBS; pH 7.4) solution at a pressure of 50 mm Hg for 30 minutes. The increase in IOP served to drive water out of the corneas, which counteracted postmortem swelling and thinned the corneas. The eyes were then fixed under pressure by perfusion with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) in PBS. Finally, the corneas were removed from the globes by an ophthalmologist, taking care to preserve a thin, 1- to 2-mm rim of sclera that was cut slightly thicker at the anatomic 12 o'clock position to indicate orientation.

The corneas were cut with a scalpel blade along the vertical meridian in superior-inferior direction approximately 1 mm off center. The larger piece was embedded vertically in low-melting-point agarose (NuSieve GTG; Lonza, Rockland, ME). Multiple consecutive slices, each approximately 250 μm thick, were cut on a vibratome (model 1500; Intracel Ltd., Shepreth, UK) and stored in PBS.

NLO Imaging. Following sectioning, the samples were placed on glass slides under a laser scanning microscope (LSM 510; Carl Zeiss Inc., Thornwood, NY) and illuminated with 150-fs laser pulses (Chameleon Titanium Sapphire laser; Coherent, Santa Clara, CA) tuned to 800 nm. Backscattered second harmonic light at 400 nm was collected by the microscope's meta-detector array.

3-D image stacks were acquired with a 40 $\times$ /1.2 NA objective (Apochromat; Carl Zeiss) with increasing focus depth at 2- μm intervals to a depth of approximately 100 μm . Each stack had a voxel resolution of $0.44 \times 0.44 \times 2 \mu\text{m}$ at $512 \times 512 \times 50$ voxels ($x \times y \times z$). To image the entire slice across the full corneal diameter, we acquired consecutive stacks with a macro function (MultiTime; Carl Zeiss). The total number of stacks varied with corneal size and curvature and was typically around 200 individual stacks. Scanning a complete segment took approximately 12 hours.

TABLE 1. Donor and Eye History, Corneal Thickness, and Study Design for All Eyes

Eye	Age (y)	Sex	Ethnicity	Time to Preservation (h, min)	Time to Experiment* (h)	Final Corneal Thickness† (μm)	Study
1	65	M	Caucasian	15, 21	36	770	HRMac
2	57	F	African American	3, 18	36h	460	HRMac
3‡	67	—	—	—	—	670	HRMac
4	34	F	Caucasian	14, 5	36h	670	HRMac
5	63	F	Asian/Pacific	4, 50	28h	480	HRMac
6	48	f	Caucasian	2, 11	24h	540	Biomechanics
7	48	f	Caucasian	2, 11	24h	560	Biomechanics
8	91	f	Caucasian	13, 54	192h	540	Biomechanics
9	69	f	Caucasian	6, 50	48h	520	Biomechanics
10	100	f	Caucasian	28, 10	168h	500	Biomechanics
11	100	f	Caucasian	28, 10	168h	500	Biomechanics
12	88	f	Caucasian	19, 45	120h	540	Biomechanics

* For HRMac corneas, time to experiment refers to the time before the corneas were fixed in PFA.

† HRMac corneal thickness was measured histologically. Biomechanical corneal thickness was measured by pachymetry.

‡ Donor information was not available for this sample.

Image Processing. Image processing, analysis, and visualization were performed on a computer (dual Xeon X5550 Quad Core CPU [Intel, Mountain View CA], clocked at 2.67 GHz per core with 24 GB of RAM running a 64-bit version of Windows 7 [Microsoft, Redmond, WA]). After imaging, the stacks were converted from native .lsm format (Carl Zeiss) and saved as a series of .tiff files using the LSM toolbox plugin for ImageJ version 1.39 (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/index.html>). Stitching was accomplished through a combination of the ImageStitch plugin and a custom-written ImageJ macro. These plugins combined the individual .tiff files into mosaics for each plane, using registration algorithms to correct for image misalignments due to sample drift or imprecise stage movements. The resulting planes were combined into a single, multiplanar .tiff file for each sample.

Mosaics were loaded into image-analysis software (Amira 5.2; Visage Imaging, Carlsbad, CA) for 3-D reconstruction. Representative collagen fibers in the anterior, mid, and posterior stroma were individually segmented with the label field module by manually highlighting them across adjacent slices. The segmentation process was started halfway through the stack for each fiber to avoid tracking fibers that moved beyond the upper and lower bounds of the stack.

Collagen Fiber Branching. Collagen fiber interconnectivity was measured by determining the density of fiber branching points at a given stromal depth. To accurately assess branching point density (BPD) in different corneas that vary in both thickness and shape, we developed a mathematical approach for collecting data relative to the shape of each cornea.

First, approximately 200 landmarks each were placed along the anterior and posterior surface of the corneal reconstruction (Meta-morph software; Molecular Devices, Sunnyvale, CA). The coordinates were logged to a spreadsheet (Excel; Microsoft, Redmond, WA) and using statistical analysis software (Origin 8; Hearne Software, Melbourne, VIC, Australia), second-order polynomial curves were fitted through the data points with a very high degree of accuracy ($R^2 > 0.99$). These polynomials were expressed as

$$f(x) = ax^2 + bx + c \quad (1)$$

where a determines the curvature of the cornea and b and c determine the relative angle and position of the cornea on the microscope stage. These polynomials provided an accurate mathematical description of the anterior and posterior surfaces of each cornea.

Because corneas are curved, relative distances had to be calculated along the curvature of the cornea rather than along straight lines. Using the arc length equation,¹⁵ the distance between two points x_1 and x_2 on the anterior stroma were expressed in terms of $f(x)$:

$$D(x_1, x_2) = \int_{x_1}^{x_2} \sqrt{1 + \left[\frac{d}{dx} f(x) \right]^2} dx \quad (2)$$

Using the coordinates of the start and end of the ALL (Bowman's layer), the length of the stroma from limbus to limbus and the position of the corneal apex were determined. To generate landmarks in the central cornea, we used the apex as a starting point for all eyes. A group of five lines perpendicular to the ALL, centered around the apex and spaced 500 μm apart, were drawn across the stroma, connecting the anterior and posterior surfaces (Fig. 1). Each line was then divided into 15 segments of equal length. Prominent fibers closest to each segment were then followed in 3-D, and the locations of the two closest branching points on either side of the line were determined and logged to a spreadsheet. By measuring the distance between branching points, an average BPD per millimeter as a function of stromal depth was obtained. For each cornea, the BPDs for the five locations at the same depth were averaged, and the mean and standard deviation were recorded for each of the 15 depth locations.

Biomechanics

Local corneal compliance was assessed based on indentation testing. This method is used to measure compliance in biological soft tissues and is a well-established way of characterizing the nanoelastic properties of various ocular tissues.^{16,17} A more detailed description of indentation testing is provided by McKee et al.¹⁸ Briefly, a sample is indented with a probe of known diameter at a constant rate, while a force transducer constantly measures the force exerted. The resulting force-indentation curves can then be used to calculate the localized elastic modulus for the tissue.

Sample Preparation. Seven eyes of five donors aged 48 to 100 years (mean, 79.2 ± 20.8 years) were prepared for femtosecond laser-based LASIK flap dissection by inserting a needle into the vitreous and infusing PBS under 80 mm Hg of pressure. The epithelium was scraped off with a scalpel, and 20% low-fraction dextran solution (Acros Organics USA, Morris Plains, NJ) was applied to the cornea at regular intervals throughout the pressurization process, to counteract post-mortem corneal swelling. Central corneal thickness was monitored with a pachymeter (IOPac Standard; Reichert, Depew, NY) until the corneas returned to a normal hydration state, indicated by a central thickness of approximately 550 μm .

Femtosecond Flap Creation. Following pressurization and thinning, eyes were placed under a surgical femtosecond laser system (Intralase; Abbot Medical Optics Inc., Abbot Park, IL) while still under pressure. Two LASIK flaps were cut at 360- and 180- μm depths with a 2.5- μJ pulse. The flaps were separated with a thin probe and placed in

FIGURE 1. To quantify lamellar branching, curve fits of Bowman's and Descemet's membranes were connected with vertical lines spaced 500 μm apart. These vertical lines were further subdivided by 15 horizontal guide lines. A prominent fiber closest to each of those guidelines was tracked in 3-D, and the location of the two closest branching points on either side was logged on a spreadsheet.

silicone oil to maintain their hydration state. The oil prevented the flaps from drying out due to exposure to air without diffusing into the flaps as a buffer solution would. The remainder of the cornea was then removed from each eye, and the final posterior corneal layer was removed with a 5-mm-diameter trephine blade. This procedure provided us with three flaps, each approximately one third of the total corneal thickness. While removing the anterior flaps, we observed that the remaining posterior stromal tissue began to swell, resulting in a 30% increase in final thickness of the posterior flap compared to the middle and anterior flaps.

Mechanical Testing. Flaps were prepared for mechanical testing by first submerging them in low-viscosity silicone oil (Clearco Products Co, Bensalem, PA) for 20 minutes to maintain their hydration state. Flaps were then transferred to a 22-mm steel washer filled with silicone oil that was mounted on the X-Y plane of a high-performance modular ball bearing linear three-stage system (460P-XYZ; Newport, Inc., Irvine, CA), allowing for 3-D positioning of a cylindrical flat-tipped 1-mm diameter probe attached to a force transducer (F10; Harvard Apparatus, Holliston, MA) mounted on the vertical plane. The X and Y stages, responsible for lateral movement, were driven by manual actuators (SM-25; Newport, Inc.). Axial position control was achieved through a third stage driven by a computer-controlled motorized actuator (LTA-HS; Newport, Inc.), via a control module (ESP 300; Newport, Inc.) and a custom-written program (LabVIEW; National Instruments, Austin, TX).

Signals from the force transducer were amplified (TAM-D; Harvard Apparatus), filtered (Power Laboratory 4/30; Harvard Apparatus) and converted to force (Labchart software; Harvard Apparatus). The probe indented the sample at a constant rate of 3 $\mu\text{m/s}$. Force and position data from the transducer and the stage were recorded every 100 ms and logged to a spreadsheet, with a force resolution of 5 μN and a spatial resolution of 0.035 μm .

This setup was used to indent the corneal flaps in three separate spots while simultaneously recording probe position and applied force. Spots were selected 1.5 mm from the center of a probe with 120° of angular separation. Between measurements, the tissue was allowed to achieve equilibrium for about 3 minutes, while the probe was being repositioned. Before each measurement, full contact between the probe and the tissue was achieved by monitoring the indentation force. A linear increase in indentation force indicates full contact between the flat end of the probe and the corneal flap.¹⁹ Force-displacement curves were generated for each spot and used to calculate the effective elastic modulus, using Hayes' equation¹⁹ which also applies to soft biologic tissue²⁰:

$$E = \frac{1 - \nu^2}{2\alpha\kappa} \cdot \frac{F}{w} \quad (3)$$

where E is the effective elastic modulus, ν is Poisson's ratio, α is the radius of the indenter, w is the indenting depth, F is the indenting force, and κ is a correction factor based on the ratio of indenter radius and material thickness taken from Hayes et al.¹⁹ Based on previous work by Liu and Roberts,²¹ a Poisson's ratio of 0.49 was assumed for corneal tissue. This equation assumes a linear elastic model, and the elastic modulus was therefore calculated for the linear range of the stress-strain curves only. Finally, the elastic modulus was averaged over all three spots and plotted as a function of indentation depth, yielding a modulus-strain curve. Poisson's effect due to stretching of the corneas was not taken into account in our study, as Jue and Maurice²² have shown that corneal expansion in our pressure range is negligible.

Statistics

One-way analysis of variance was performed on the BPD and elastic modulus datasets according to the Holm-Sidak method (SigmaStat 3.1.1; Systat Software, Inc., San Jose, CA).

RESULTS

A low-resolution (3.2 $\mu\text{m}/\text{pixel}$), single-plane HRMac image of a 120- μm -thick section from the central corneal meridian cut in superior-inferior direction is shown in Figure 2. This sample has a corneal arc length of approximately 12 mm. The original HRMac scan is 25,000 $\times$ 9,000 pixels for each of the 60 planes at a resolution of 0.44 $\mu\text{m}/\text{pixel}$ and is made up of more than 80,000 individual images. Figures 2B and 2C show views of the central portion of the cornea at increasing resolution (1.14 $\mu\text{m}/\text{pixel}$ and 0.44 $\mu\text{m}/\text{pixel}$), revealing complex interactions between collagen fibers.

Three-Dimensional Visualization

Selected fibers were reconstructed in 3-D using the surface render module to qualitatively assess the degree of fiber interconnectivity. Branching occurs three-dimensionally; in some cases, fibers continue laterally after branching, whereas others branch in the anterior-posterior direction. Figure 3 shows 3-D views of several fibers from the anterior, mid, and posterior stroma. The anterior fibers (Fig. 3B) show a markedly higher degree of branching than the fibers in the mid (Fig. 3C) stroma, whereas those in the posterior part of the stroma (Fig. 3D) exhibit almost no branching at all.

3-D reconstructions also identified two other distinct collagen fiber structures: (1) long, prominent fibers originating at or near the limbus that extend for several millimeters across large portions of the cornea (Fig. 4A, green fibers, arrow). These

FIGURE 2. (A) Single-plane, zoomed-out HRMac image of a full-diameter corneal cross section along the vertical meridian. The full scan is composed of 60 such planes and is made up of over 80,000 individual images with a total size of 25,000 $\times$ 6,500 pixels per plane, shown here at a resolution of 3 $\mu\text{m}/\text{pixel}$. (B) A zoomed-in view of the central portion having a resolution of 1 $\mu\text{m}/\text{pixel}$. At the bottom, Descemet's membrane has become detached near a fold that is most likely the result of postmortem swelling. (C) The anterior central cornea at full resolution (0.44 $\mu\text{m}/\text{pixel}$). Note the presence of the ALL (white arrow) and the insertion of collagen fibers (black arrowheads).

FIGURE 3. (A) Section from a single HRMac plane of the central cornea overlaid with 3-D reconstructions of representative fibers. Representative fibers were manually segmented and rendered. Shown are fibers at different stromal depths, enlarged views of which are shown in (B) through (D). Three-dimensional surface renders of selected fibers from the anterior (B), mid (C), and posterior (D) stroma highlight the drop in branching complexity with increasing stromal depth.

fibers do not follow the corneal curvature from limbus to limbus; instead, they seem to traverse many layers before terminating at or near the ALL (Bowman's layer).² Bow spring-like fibers originate from the highly intertwined layers directly beneath the ALL and arc upward, fusing with the ALL before arcing back down (Figs. 4B, 4C, blue fibers, arrows). Bow spring fibers are characterized by a near-parabolic shape, the apex of which is fused with the ALL. These interactions are best observed in 3-D simulations, as shown in Supplementary Movie S1 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8070/-/DCSupplemental>).

Branching Point Density

Collagen fiber interconnectivity, expressed as a function of BPD versus stromal depth, is shown in Figure 5. Plots for all corneas (Fig. 5A) show considerable variation in total BPDs between individual corneas; however, all corneas exhibit a nonlinear decrease in BPD with increasing distance from the ALL. Data from all five corneas were averaged (Fig. 5B), and, based on the nature of the decrease in BPD, an exponential curve was fitted to the data with a high degree of accuracy ($R^2 > 0.98$), using the equation for the fitted curve,

FIGURE 4. 3-D reconstruction of bow spring fibers (blue), anchoring fibers inserting from the limbus (green), and the highly intertwined anterior fiber meshwork (teal) near the ALL (gold).

FIGURE 5. (A) Individual branching point densities as a function of stromal depth. Each line represents the BPD for a single eye. (B) BPD averaged over all five eyes as a function of stromal depth. *Solid line*: an exponential curve fit with $R^2 > 0.98$. (C) BPD averaged over five HRMac-imaged corneas. BPD values from across the stroma were grouped into three layers to facilitate comparison with biomechanical properties. NS, no statistically significant difference between values ($P = 0.05$).

$$BPD(z) = -7.766 \cdot \ln(z) + 39.084 \quad (4)$$

where z is the stromal depth measured in percent, the data can be interpolated to determine BPD at points of interest throughout the stroma. Compared to an initial BPD of 26.6/mm just

below Bowman's layer at 5% stromal depth, the BPD drops to approximately 8.7/mm halfway through the cornea, representing a 66% drop in collagen fiber interconnectivity.

To compare these results with biomechanical data from the remaining eyes, the data were grouped into anterior, mid, and posterior layers, each comprising one third of total stromal thickness (Fig. 5C). The anterior third is significantly more intertwined than the middle ($BPD_{\text{anterior}}/BPD_{\text{mid}} = 2.05$, $P < 0.01$) and the posterior thirds ($BPD_{\text{anterior}}/BPD_{\text{posterior}} = 3.5$, $P < 0.01$). There was no statistically significant difference between the middle and the posterior stroma ($BPD_{\text{anterior}}/BPD_{\text{posterior}} = 1.6$, $P = 0.05$).

Mechanical Testing of Corneal Flaps

Seven eyes of five donors were evaluated. The sample quality varied between donors, as some eyes had been refrigerated for up to a week before being transferred from the eye bank. These eyes exhibited severe swelling and had to be thinned for more than 2 hours before the laser cuts were made. In this study, we found no clear relationship between donor age or sample storage time and mechanical properties after thinning the corneas, although it should be noted that due to the comparatively small sample size, the lack of a relationship is not necessarily indicative of a lack of an overall trend.

Mechanical testing generally showed that the anterior flap was markedly stiffer compared with the other two flaps. Strain-modulus curves are shown in Figure 6A and 6B for two different corneas. Both total strain and the shape of the curve varied between eyes. In some cases, the stiffness curve assumed a parabolic shape (Fig. 6A). Overall, stiffness increased as the strain approached 10% (Fig. 6B) in most samples. The linear range of the stress-strain curves varied between eyes; however, all curves remained linear up to at least a 6% strain. Since our equation is based on the assumption of a linear elastic model, elastic moduli were calculated for the 6% strain range. Figure 6C shows average strain-modulus curves using data from all seven eyes. The anterior flap stiffness is much higher than that of the middle and posterior flaps, and increases with higher strains.

Averaging over the strain-modulus curves yielded mean elastic modulus values for each flap (Table 2). The elastic modulus of flaps varied considerably between individual eyes, as did the slopes of these curves. Flap thickness varied between eyes as well (anterior, $140 \pm 41 \mu\text{m}$; mid, $199 \pm 46 \mu\text{m}$; and posterior $263 \pm 58 \mu\text{m}$), but there was no apparent connection between flap thickness and the slope of the curve. Thickness measurements before and after indentation tests showed that indenting the flaps did not change the thickness permanently (mean $\Delta_{\text{thickness}}$, $10 \mu\text{m}$). Averaging over all seven eyes (Fig. 6D) showed that the anterior flap was significantly stiffer than the middle ($E_{\text{anterior}}/E_{\text{mid}} = 1.9$, $P < 0.008$) and the posterior ($E_{\text{anterior}}/E_{\text{posterior}} = 2.2$, $P < 0.003$) flaps. There was no significant difference between the middle and the posterior flaps ($E_{\text{anterior}}/E_{\text{posterior}} = 1.18$, $P = 0.6$).

To investigate the effects of swelling on corneal elasticity, we first tested the second (midstromal) flap from an eye, following the standard protocol. The flap was then submerged in PBS and allowed to swell for 20 minutes. During this time, flap thickness increased from 160 to $354 \mu\text{m}$. At this point, the flap underwent mechanical testing a second time. Figure 7 shows a marked increase in flap rigidity as a result of swelling.

DISCUSSION

In this study we visualized and quantified the axial heterogeneity in corneal collagen fiber organization and its potential effects on corneal biomechanics using a combination of NLO-

FIGURE 6. Representative strain-modulus curves from two eyes. (**A**, **B**) The variation in effective elastic modulus. Depending on the hydration state, a swollen posterior flap can appear stiffer than the middle flap (**A**). Some corneas exhibited a strong increase in stiffness with increasing strain (**B**). An average plot of all seven corneas also shows this increase (**C**). The anterior flap is markedly stiffer than the middle flap in all samples. (**D**) Elastic modulus averaged over all seven corneas grouped by flap position. NS, no statistically significant difference between values ($P = 0.6$).

HRMac imaging and mechanical testing. Collagen fibers were visualized in 3-D, and fiber interconnectivity was measured as a function of stromal depth. Our results show that collagen fiber patterns are much more complex than conventionally depicted and that there is a significant axial heterogeneity in the interconnectivity of these fibers. We were also able to identify new collagen fiber types—bow spring and anchorlike fibers—which, to the best of our knowledge have not been reported. Finally, we show that the anterior corneal stroma has a significantly increased effective elastic modulus compared with that of the middle and posterior stroma. Although specific measurements of fiber branching and elasticity were not made on the same cornea, these findings strongly suggest that the collagen fiber interaction may influence the biomechanical properties of the cornea.

Different forms of corneal axial heterogeneity have been reported by multiple investigators using a variety of imaging

modalities. Abahussin et al.²³ describe a decrease in preferred collagen orientation in the anterior cornea using x-ray diffraction imaging. Swelling studies conducted by Müller et al.²⁴ showed that corneal swelling is limited to the posterior and middle stroma, suggesting a structural and biomechanical heterogeneity. Transmission electron microscopy images show a larger degree of interconnectivity between fibrils in the anterior stroma,²⁵ and that the collagen fibrils that make up the larger fibers occasionally bifurcate.⁵ Finally, on a cellular level,

TABLE 2. Elastic Modulus for the Three Flaps in the Seven Eyes that Underwent Indentation Testing

Eye*	Anterior	Mid	Posterior
6	1861.7 ± 447.3	854.3 ± 265.5	649.8 ± 178.6
7	891.9 ± 712.3	666.9 ± 330.9	706.5 ± 158.5
8	2583.4 ± 1067.7	1240.8 ± 241.3	364.5 ± 181.2
9	938.8 ± 469.7	336.9 ± 117.6	1273.4 ± 392.9
10	1442 ± 263.5	1000.9 ± 351.6	469.4 ± 187
11	1730.5 ± 649.2	594.2 ± 383.2	764.4 ± 453.9
12	942.3 ± 424.3	828.3 ± 56.3	438 ± 96

Data are expressed in mean Pascals ± SD. See Table 1 for donor information and eye history.

FIGURE 7. Modulus-strain curves from a single midstromal flap show the effects of swelling on corneal rigidity. This flap underwent regular compression testing before being allowed to swell for 20 minutes, resulting in a twofold increase in flap thickness and a threefold increase in elastic modulus.

confocal microscopic studies show an axial heterogeneity in keratocyte density, which has been found to decrease with increasing stromal depth in both rabbit²⁵ and human corneas.²⁶

Overall, our findings are consistent with these earlier reports of heterogeneity¹⁴ and indicate that collagen organization is highly complex, showing a logarithmic decrease in collagen fiber interconnectivity with increasing stromal depth. This axial heterogeneity is the result of a marked difference in collagen fiber patterns, which change gradually with stromal depth. As a result, fewer fibers are aligned along the preferred meridians, which would cause a drop in preferred orientation on x-ray diffraction images.²³ At the posterior end, near Descemet's membrane, fibers run in parallel almost entirely uninterrupted for several millimeters. These patterns are consistent with the classic model described in the literature of orthogonally arranged sheets of fibers.^{27,28} About halfway through the stroma, collagen fibers branch more frequently. However, most fibers follow the curvature of the cornea and run for long distances. Overall, this area appears to be a transition zone between the posterior third and the highly complex anterior third. Anteriorly, the amount of interconnectivity markedly increases. Most fibers appear to branch and fuse with several other fibers. Contrary to the classic model of interwoven bands of collagen, we observed true intertwining. Fibers split and joined other fibers, suggesting that individual fibrils contribute to multiple fibers, which may be running in parallel to the original fiber or may move off at an angle. The branching and fusing patterns on the fibrillar (nano-) scale that are visible on electron microscopy images are mirrored on a microscopic level, where they result in the intertwining of fibers seen in this study. This intertwining links fibers much more tightly than mere interweaving.

The insertions into the ALL observed in this study have been reported as early as 1849. Drawings of light microscopic sections by Bowman showed the presence of collagen fibers extending from the ALL into the stroma, where they appeared to be fusing with fibers following the corneal curvature.²⁹ More recently, transmission electron microscopy studies have shown insertion of collagen fibers into the ALL at electron-dense plaques, with short extensions into the underlying stroma.²⁵ In this study, we observed fibers extending upward from the densely intertwined meshwork just beneath the ALL, arcing upward and fusing with the ALL. In some cases, these fibers descended again and fused with other collagen fibers, forming a near-parabolic arc. These bow spring fibers are highly reminiscent of the load-bearing elements of girders and bridges. This population of fibers seems to exist in addition to the sutural fibers described by Morishige et al.,¹⁰⁻¹² which insert into the ALL and terminate there, rather than arcing and fusing with the meshwork underneath the ALL. It is conceivable that bow spring fibers represent a variation of sutural fibers and that they serve a similar function.

In addition, we observed fibers that inserted from the limbus about midway between Bowman's and Descemet's layer, and rather than following the curvature of the cornea, ran in a nearly straight line for several millimeters before branching and fusing with the meshwork just underneath the bow spring fibers. These anchorlike fibers directly connected the most highly intertwined areas of the cornea with the limbus and bear a resemblance to the anchoring fibers described by Aghamohammadzadeh et al.,⁶ which also insert from the limbus and stretch out toward the central cornea. Their connection to the meshwork underlying the bow spring fibers suggests that they serve an anchoring function.

Our data further show that the degree of collagen fiber intertwining was linked to local stromal elastic modulus. The highly intertwined anterior third of the cornea was signifi-

cantly stiffer than the much less intertwined posterior two thirds. This observation matches data published by Kohlhaas et al.³⁰ obtained through extensometry measurements of corneal strips, which also show a stiffer anterior stroma. Interestingly, despite using a vastly different approach and measuring tensile rather than compressive modulus, they also reported a ratio of approximately 2:1 between anterior and posterior flap rigidity.

Although the decrease in elastic modulus between the anterior and middle flaps matched the decrease in BPD, the posterior flap remained almost as stiff as the middle flap. In isolated cases, the effective elastic modulus even surpassed that of the anterior flap. This behavior is most likely due to the effects of postmortem swelling. As the endothelial pump shuts down after death, the cornea becomes excessively hydrated and swells. This swelling occurs primarily in the posterior portion of the stroma,³¹ and as a result the posterior flap contains considerably more water than the anterior and middle flaps. Swollen flaps are markedly more rigid than thinned flaps (Fig. 6), which explains the increase in elastic modulus for the posterior flaps. The current approach to normalizing hydration is somewhat imprecise and, due to the effect of hydration on corneal rigidity, is likely to introduce artificial variations in measured elastic modulus between individual corneas and perhaps even between different regions of the same flap. Future studies should seek to improve this method. However, it should be noted that since swelling is mostly limited to the posterior portion, the overall hydration state after thinning should have a limited effect on the anterior cornea.

Swelling studies conducted by Müller et al.²⁴ show increased resistance to swelling, even in extreme hydration states for the anterior stroma. This behavior can be explained by the much higher degree of anterior intertwining that is visible on HRMac images. Intertwining of fibers stabilizes the cornea by mechanically linking neighboring layers, making it more difficult for interfibrillar spacing to increase. Since the tensile strength of a collagen fiber is greatest along its long axis,³² fibers that branch off and fuse with others form strong links between layers, vastly increasing axial stiffness and the force required to separate adjacent layers. Conversely, in the posterior stroma the amount of intertwining is minimal, thereby allowing fibers to swell in reaction to the movement of water into the interfibrillar space, their expansion being only minimally inhibited by the presence of neighboring fibers.

With these findings, we can enhance the current model of corneal collagen organization. Using the comprehensive model described by Meek and Boote¹ as a basis, we propose the following additions: (1) Bands of parallel collagen fiber bundles traversing the entire width of the cornea are limited to the posterior stroma. With decreasing distance from the ALL, the degree of fiber branching increases exponentially, resulting in a densely intertwined meshwork of fibers in the anterior stroma that exhibits less alignment along preferred directions. This structural heterogeneity matches the axial heterogeneity in elastic modulus; highly intertwined areas are more rigid than the mostly parallel fibers that make up the posterior portion of the stroma. (2) Anchorlike fibers inserting from the limbus, rather than following the curvature of the cornea at a fixed depth, traverse several layers and terminate in a network of smaller branches in the central cornea near the ALL. These fibers create mechanical links between the limbus and the central cornea and may help distribute loads. In addition, they connect spatially separate fibers across multiple layers, which prevents slipping of layers and may serve to counteract swelling forces in the middle stroma. (3) Further stabilization of corneal shape is achieved by means of interactions between the anterior stroma and the ALL in the form of sutural or bow spring fibers, which themselves are connected to the lower stromal regions and to the limbus through anchoring fibers.

The lack of bow spring fibers in keratoconus corneas, which do not maintain normal curvature, strongly suggests that these fibers play an important role in maintaining corneal shape.¹¹

These novel structural details regarding corneal collagen organization suggest that the intertwining of collagen fibers plays a role in local variations of corneal curvature and that overall corneal shape, which is a function of corneal rigidity, is ultimately controlled by collagen fiber interconnectivity. Densely intertwined regions of the cornea are more rigid and resist the outward force resulting from IOP more so than less densely intertwined areas. As such, it would be expected that stiffer regions with higher degrees of intertwining would react differently to IOP and subsequently have different local radius of curvature than the less intertwined regions. These variations may explain deviations in corneal topography and why some areas are steeper than others. Changes in corneal curvature after local stiffening have been observed in keratoconus corneas after corneal cross-linking treatment.³³ Wollensak³⁴ observed local flattening in areas of keratoconus corneas with increased rigidity after undergoing UV cross-linking treatment compared to the untreated, structurally compromised parts of the cornea. These findings are consistent with the hypothesis that regional stiffening of the anterior cornea, through collagen intertwining, controls corneal shape, although the exact relationship between anterior corneal stiffening and corneal curvature has yet to be elucidated.

Axial heterogeneity in fiber intertwining and mechanical rigidity also has important implications for understanding the effects of refractive surgery. The two most popular methods, LASIK and PRK, reshape the cornea by removing or progressively ablating the anterior stroma. If anterior corneal stiffness influences corneal shape, then procedures extending deeper into the cornea may affect curvature to a greater extent than is accounted for by simple linear models. In addition, individual variations in anterior branching and stiffness may influence the achieved refractive results of a given procedure on an individual patient basis. Taken together, our findings on the axial heterogeneity in collagen fiber branching and mechanical stiffness may therefore help to explain, in part, some of the individual differences in achieved versus predicted outcomes after these procedures. Differences in fiber branching between eyes may also underlie the susceptibility to post-LASIK ectasia, since the minimum safe thickness of unaffected tissue may be controlled by collagen fiber interactivity.

Overall, our results show that there is a link between the arrangement of corneal collagen fibers and the compliance of the cornea. Our findings further suggest that heterogeneity of collagen fiber intertwining may regulate corneal rigidity and, by analogy, corneal shape. However, future studies are needed to identify the influence of collagen fiber branching and mechanical stiffness on corneal shape, how shape is affected by refractive surgery, and how best to assess the effect clinically, perhaps by using axial biomechanical testing.

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