Biomechanics of the Anterior Human Corneal Tissue Investigated with Atomic Force Microscopy

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PURPOSE. To investigate the biomechanics of the anterior human corneal stroma using atomic force microscopy (AFM).

METHODS. AFM measurements were performed in liquid on the anterior stroma of human corneas, after gently removing the epithelium, using an atomic force microscope in the force spectroscopy mode. Rectangular silicon cantilevers with tip radius of 10 nm and spring elastic constants of 25- and 33-N/m were used. Each specimen was subjected to increasing loads up to a maximum of 2.7 μN with scan speeds ranging between 3- and 95-μm/s. The anterior stromal hysteresis during the extension-retraction cycle was quantified as a function of the application load and scan rate. The elastic modulus of the anterior stroma was determined by fitting force curve data to the Sneddon model.

RESULTS. The anterior stroma exhibited significant viscoelasticity at micrometric level: asymmetry in the curve loading-unloading response with considerable hysteresis dependent both on the application load and scan rate (P < 0.01). The mean elastic modulus ranged between 1.14 and 2.63 MPa and was constant over the range of indentation depths between 1.0 and 2.7 μm in the stroma.

CONCLUSIONS. At microscale level, the mechanical response of the most anterior stroma is complex and nonlinear. The microstructure (fibers' packing, number of cross-links, water content) and the combination of elastic (collagen fibers) and viscous (matrix) components of the tissue influence the type of viscoelastic response. Efforts in modeling the biomechanics of human corneal tissue at micrometric level are needed. (Invest Ophthalmol Vis Sci. 2012;53:1050–1057) DOI:10.1167/iovs.11-8720

The mechanical properties of biological tissues are essential to their physiological function. Most of the biological tissues are viscoelastic materials that exhibit hysteresis and reveal other time-dependent or rate-sensitive stress-strain relations, including that the stress-strain relationship will change as the loading speed changes. Moreover, they stiffen as they are strained (stress-stiffening behavior), thereby preventing large deformations that could threaten tissue integrity.

The corneal tissue is characterized by all the typical features of soft biological materials: nearly incompressible, nonlinear elastic behavior and viscoelasticity. Hysteresis is a viscoelastic property of the corneal tissue characterized by the difference in behavior in loading and unloading conditions. It represents a combination of biomechanical properties of elasticity and viscosity of the corneal tissue. The phenomenon of hysteresis in the corneal tissue is mainly due to its internal structure. Microscopic investigations revealed how the stroma is structured in several overlapping collagen lamellae composed of bundles of collagen fibrils surrounded by a jellylike matrix mostly composed of glycoproteins. The microstructure of the stroma, however, is highly heterogeneous, depending on the specific region and corneal layer being evaluated. The anterior central stromal lamellae are more closely packed than are other regions of the cornea; moreover, the anterior stroma is less hydrated than the posterior stroma with stronger junctions between collagen lamellae. It has been postulated that anisotropy in stromal architecture also results in mechanical anisotropy and that the anterior stroma, according to its architecture, holds a main importance in maintaining the corneal strength and hence curvature. This hypothesis was supported by experimental and clinical studies.

So far, most of the research on corneal mechanics has been focused on the overall bulk mechanical properties of the corneal tissue. On the other hand, a detailed knowledge of the mechanics of different corneal layers at a micrometric scale of observation could be valuable in improving our understanding of the mechanical anisotropy of the tissue and aid in the design and development of enhanced bioengineered corneal tissues.

Atomic force microscopy (AFM) has become popular in biomedical sciences as a tool for investigating topographic and mechanical properties of biological and biosynthetic materials. Indentation using AFM is evolving as a powerful tool for studying the local micro- and submicromechanical properties of a variety of biological tissues and biomaterials. The scope of our study was to provide a quantitative biomechanical measurement of the most anterior part of the human corneal stroma using AFM.

MATERIALS AND METHODS

Four fresh human donor corneas were obtained from the Veneto Eye Bank Foundation (Venezia Zelarino, Italy). The corneas were explanted between 6 and 10 hours after death and immediately preserved at 4°C in corneal storage medium (Eusol-C; Al.chi.mi.a srl, Padova, Italy) enriched with 15% dextran (molecular weight, 500). Each cornea was soaked in deionized water. The whole cornea, devoid of epithelium, was preserved in corneal storage medium enriched with 15% dextran and used for experiments within 12 hours. Corneal thickness and...
endothelial cell density were measured using ultrasound contact pachymetry (UP-1000; Nidek Co. Ltd., Gamagori, Japan) and an inverted optical microscope (Axiovert 25; Carl Zeiss Microscopy, Jena, Germany), respectively.

Before the experiment, each cornea was gently placed on a specially designed Teflon environmental cell with the endothelial side down. Mechanical properties of the tissue were measured with an atomic force microscope (NanoScope III; Veeco, Sunnyvale, CA) in the force spectroscopy mode.\(^{16}\) Measurements were performed at 32°C in storage medium-15% dextran solution, using phosphorus doped rectangular silicon cantilevers of a nominal elastic constant between 20- and 80-N/m (Newton/meter; MPP-11,120-10; Veeco, Sunnyvale, CA). The nominal value of the tip’s radius of curvature was 10 nm. Force curves were obtained on different locations at the center of the stromal surface of each sample and hundreds of force curves (no less than 50 curves for each rate) were taken at scan rates ranging between 3- and 95-μm/s at each location.

A crucial aspect in the AFM data interpretation is the correct calibration of both the cantilever elastic constant and the cantilever deflection. Before the force measurements on each sample, the spring constant \((k)\) of the cantilever was calibrated by recording the cantilever resonance frequency in air and measuring, by optical microscopy, the cantilever width and length, according to the method proposed by Sader et al.\(^{17,18}\) and Burnham et al.\(^{19}\) From these experimental measurements, the spring constants of the four cantilevers used in the experiment (one cantilever per sample) were 25 N/m (used in two samples) and 33-N/m (used in the remaining two samples). To calibrate the cantilever deflection, right before each measurement, we acquired a reference force measurement on the hard surface of a glass slide with the tip completely immersed in storage medium-15% dextran solution.\(^{20}\) We made sure that the same conditions were maintained during the experiment. At the end of every set of force measurements, we acquired an additional reference force measurement on the hard surface to verify that the calibration was not changed during the experiment. The photosensitive detector sensitivity was determined as the slope of the force curve, taken in the storage medium-15% dextran solution, when the tip was in contact with a rigid glass surface, and this value was used to convert the AFM raw deflection data in volts to deflection in micrometers \((Z_d)\) by using a custom routine (Matlab software ver. 7.0; The Mathworks, Inc., Natick, MA).\(^{20}\) Then the force profile was determined by \(F = k \cdot Z_d\), and the resultant force curve was plotted against the indentation \((\delta)\) which is given by the sum of \(Z_d\) and the piezo position \(Z_p\).

Hysteresis \((H)\) was calculated as the area encircled between the extending and retraction curves, as illustrated in Figure 1. The elastic modulus \((E)\) was calculated by fitting the Hertz-Sneddon model\(^{21,22}\) for conical indenter to force curve data, leaving as variable fit parameters the contact point and baseline (Fig. 2)\(^{22}\):

\[
F = F_0 + \frac{2}{3} \frac{E}{1 - \nu^2} (\delta - \delta_0)^2 \tan(\alpha)
\]

where \(F\) is the loading force (in micronewtons), \(\nu\) is the Poisson’s ratio (assumed to be 0.49), \(E\) is the elastic modulus (in Pascals), \(\delta\) is the indentation depth (in micrometers), and \(\alpha\) is the half opening angle of the tip (15° as specified by manufacturer); \(F_0\) and \(\delta_0\) are the loading force at baseline and the indentation depth at the contact point, respectively.

Immediately after the mechanical measurement, topographic imaging was performed on the anterior stromal surface, with the sample completely immersed in storage solution (Eusol-C; Veeco) without dextran, with an atomic force microscope (AutoProbe CP; Veeco) in contact mode.\(^{23,24}\) V-shaped silicon nitride cantilevers with a spring constant of 0.58 N/m (Veeco) and a tip radius of 10 nm were used. Images were acquired with a 256 × 256-point resolution with a scan rate of 1 Hz per line. A set of images was taken from different areas close to the center of the corneal surface of the single specimen. The area scanned was limited to a maximum of 30 μm². All the images used for roughness measurement were processed and analyzed using the specific AFM software (ProScan 1.5; Veeco).

**Statistical Analysis**

Data are expressed as the mean ± SD. The one-way analysis of variance (ANOVA) test was used to statistically compare the differences in hysteresis between the various scan rates in each corneal sample and the differences in hysteresis and modulus of elasticity between the four samples. Differences with a \(P \leq 0.05\) or less were considered statistically significant. The coefficient of variation (CoV) was calculated to estimate variability in hysteresis values between specimens. A commercial software program (KyPlot; KyensLab, Inc., Tokyo, Japan) was used for all statistical testing.

**RESULTS**

The mean donor age of the four tissue samples used for the experiment was 68.5 ± 6.0 years; the mean postmortem time, 7.7 ± 1.7 hours; the mean central corneal thickness, 515 ± 72 μm; and the mean endothelial cell density, 2063 ± 250 cells/mm².

To verify the reliability of the storage protocol and accordingly to avoid swelling or dehydration of corneal tissues during the experimental study, a control experiment was performed: ultrasound central corneal thickness measurements were performed, after the epithelium was removed, on four samples (not included in the measurements’ analysis) immersed in 15% dextran-storage solution (Eusol-C; Al.chi.mi.a. srl) for 72 hours. The mean stromal central thickness (± SD) at baseline and 72 hours later was 551 ± 59 and 522 ± 48 μm, respectively (Wilcoxon signed rank test; \(P > 0.05\)).

Each cornea was subjected to increasing loads up to a maximum of 2.7 μN, at various application rates. Force data were divided into five groups according to the application rate (3–9, 10–19, 20–29, or 30–39 μm/s and faster than 40 μm/s). No adhesion between the tip and the sample surface was detected in any sample. We made sure to avoid any possible alteration of the tissue during measurement: Any rupture in the material would have induced a discontinuity in the force curve profile and therefore would have been clearly seen.

For each group of application rate tested, hysteresis increased nonlinearly with increasing pressure loading in all the samples (Fig. 3). For application loads higher than 0.9 μN, hysteresis was strictly dependent on the application rate \((P < 0.01)\). The mean hysteresis values increased up to scan rates of 30 to 39 μm/s, then decreasing at scan rates faster than 40 μm/s. Mean hysteresis values are summarized in Table 1. \(H\) values ranged from a minimum of 0.147 to 0.257 pJ for an application load of 1.0 ± 0.1 μN at scan rates slower than 10 μm/s to a maximum of 0.564 to 0.756 pJ for an application load of 2.0 ± 0.1 μN at scan rates of 20 to 29 μm/s. No statistically significant differences in \(H\) values between samples were found. For the highest application loads of 1 and 2 μN \((P > 0.05)\). In corneal sample 2, hysteresis was fairly constant at 1.0 ± 0.1 μN load over the range of scan rates. The variation in hysteresis values among specimens was lower at a 2-μN load (CoV, 11%) than at 1.0 μN (CoV, 20%).

The Young’s modulus of elasticity \((E)\) was calculated for each cornea individually. \(E\) was calculated by fitting the Hertz-Sneddon model to force curves taken at application loads higher than 0.6 μN and scan rates lower than <9 μm/s. (Below 0.6-μN loads, the Sneddon model did not fit the data accurately.) \(E\) was constant over the range of indentation depths in all the tissues, as shown in Figure 4: mean \(E\) value was 1.14 ± 0.03, 2.63 ± 0.17, 1.33 ± 0.08, and 2.26 ± 0.07 MPa in corneal samples 1, 2, 3, and 4, respectively (\(P < 0.001\)). Maximum
indentation depth was 2.55, 1.92, 2.74, and 2.34 μm, respectively.

The anterior stromal surface showed a feltlike appearance, with numerous pores of various depths and dimensions (Fig. 5), demonstrating typical Bowman’s layer characteristics. The surface roughness estimation was performed in each corneal sample by calculating the root mean square value of the roughness within the given area (RMS roughness; i.e., the standard deviation of the height data, on 20 reference surface areas of 10 μm²: the mean RMS roughness was 102 ± 7 nm.

**DISCUSSION**

The human corneal structure is dominated by the stroma, which shows a composite layered structure formed by hundreds of collagen lamellae that are, on average, 2 μm thick, embedded in a hydrated matrix of proteoglycans and glycosaminoglycans that run parallel to the surface of the tissue. Collagen lamellae are the main load-bearing elements of the stroma and are assumed to determine the anisotropic and highly nonlinear viscoelastic behavior of the cornea. Anisotropy in stromal architecture caused a mechanical anisotropy of the tissue: Meridional and depth-dependent variations in the mechanical properties of stromal constituents have been discussed in several publications. The viscoelastic response of the corneal tissue has been widely characterized at the macroscopic level. The results of these works have shown some differences, suggesting that there are various external factors, such as the rate of storage media, measurement temperature, humidity, swelling or dehydration of the tissue, testing techniques, and protocols that may affect the viscoelastic response of the corneal tissue in the experimental environment. On the other hand, all experiments have shown that the nonlinear stress-strain (J-shape) response of the cornea (in porcine, bovine, and human eyes) is rate dependent. Hysteresis in general increases with faster pressure applications. In healthy eyes, corneal hysteresis was found to significantly increase with greater central corneal thickness and decrease with greater age and higher intraocular pressure. Hysteresis, as measured by a commercial device, is on average lower in eyes with corneal disorders than in normal eyes. On the other hand, uncertainties as to the precision of assessments of the viscoelastic response of corneal tissue performed with commercial instruments remain.
Work is needed to understand corneal hysteresis in detail and to develop reliable methods to quantify this property in the clinical setting. Because of the intrinsic local variations in microstructure and biomechanics of the stroma, investigation of the tissue’s properties at microscale level could be preferable. In this study, we sought to characterize the biomechanical response of the most anterior part of the stroma with atomic force microscopy. Testing was conducted with the cornea covered in a bath of 15% dextran-enriched storage solution. This procedure avoided any tissue swelling during the experiment. In addition, the storage medium has been demonstrated to maintain the corneal thickness within the physiological range and has been successfully used for mechanical testing of human corneas.

Human corneas were probed with a nanometer-sized tip, and AFM data were analyzed for application loads higher than 0.6 μN taken at various scan rates ranging between 3- and 95 μm/s. We were able to indent the anterior stroma up to a 2.7-μm depth characterizing the mechanical behavior of the most anterior collagen lamella under Bowman’s layer, further avoiding damage to the stromal surface. The viscoelastic response was comparable between tissues: Hysteresis increased nonlinearly with increasing pressure application and was highly rate dependent (increasing up to scan rates of 30 to 39 m/s, then decreasing at scan rates faster than 40 m/s), showing lower values at slow scan rates. The range of variation in hysteresis values between samples (expressed by CoV) was between 10% and 20%, depending on the application load: a higher variability was measured at lower loads.

![Figure 2](https://example.com/Figure2.png)

**Figure 2.** (A) The corneal surface indentation by the AFM tip. Shown is the bending of the cantilever at the contact point (top tip) and during indentation (bottom tip). At the contact point the indentation (δ) is 0; at increasing loading, δ is calculated by adding the cantilever deflection (Zc) to the piezo displacement (Zp). (B) To measure the material properties of the tissue, the spring’s elastic constant of the cantilever must be comparable or higher than that of the sample probed. If the surface is hard (e.g., a glass slide in our experiment) there is no measurable indentation of the tip on the surface, and the movement of the tip and the piezo scanner is identical (represented by the straight interrupted line). When the tip does indent the surface of the cornea, however, the slope of the curve will be less than that measured at a hard surface and the mechanical properties of the tissue can be determined. Zc and Zp are adjusted to derive the tip-sample separation (force-indentation curve). (C) Curve fit of the experimental data with the Sneddon equation to determine Young’s modulus of elasticity (E). The Sneddon model assumes elastic behavior of the sample; on the other hand, the energy delivered by the indenter is not completely given back by the corneal tissue but dissipates owing to its viscoelastic behavior that also appears as hysteresis between the extend and the retract part of the force curve (Fig. 1). To obtain optimum and reproducible results for elasticity calculations, it is recommended that E be fit for each point of the force-indentation curve where the geometry of the indentation matches the geometry of the indenter. At the maximum indentation E can be considered a result of the substrate stiffness.

![Figure 3](https://example.com/Figure3.png)

**Figure 3.** Plot of hysteresis values in function of the applied load in one central location of sample 3. Hysteresis increased nonlinearly with increasing application loads and, at the same time, was influenced by the application rate. The anterior stromal hysteresis increased between scan rates of 3 and 39 μm/s; for scan rates higher than 40 μm/s, H decreased toward values measured at the lowest scan speeds.
The anterior stroma of corneal sample 2 showed less hysteresis than other samples and was found to hold the stiffest anterior stroma (i.e., the highest $E$ value). Differences in the mechanical behavior between tissues could be related to the stromal microstructure and ultimately to the complex combination of elastic and viscous components in the stroma and its water content. Collagen fibrils are highly hydrated and are surrounded by a jellylike matrix mostly made of proteoglycans and glycosaminoglycans; moreover, the presence of intra- and intermolecular cross-links between collagen fibers may influence the viscous stretching of the lamellae and hence the stromal toughness. The microstructure of the tissue (e.g., number of cross-links, nature of the ground substance, water content, age, and stromal degeneration) also influences the relation between elasticity and hysteresis. Investigators modeled how changes in elasticity influence hysteresis measurement in a complex and nonlinear way: Hysteresis can be associated with either high or low elasticity and can increase or decrease with stiffening of the cornea according to microstructure. At AFM imaging, although the stromal surface roughness was comparable between tissues, the surface topography of sample 2 showed a higher density of pores and thinner fiber bundles than the other samples. Differences in surface morphology may reflect comparable differences in the underlying anterior stromal microstructure, with more densely packed collagen fibers and therefore a stiffer cornea.

As discussed above and in other studies, we reasonably assumed that at a slow loading rate ($<9 \mu m/s$) relative to the viscoelastic regimen experimentally observed, the microindentation response under loading was mainly determined by the elastic properties of the tissue. $E$ values were constant over the range of indentation depths (from 1 to 2.7 $\mu m$) in all tissues, indicating homogeneity in the local microstructure and mechanics of the most anterior portion of the tissue. This may imply that we were capable to indent and therefore measure the mechanical properties of the most anterior collagen lamella underlying Bowman’s layer. In the literature, the range of variation for the human corneal modulus of elasticity is between 0.5 and 57 $MPa$. Variation in $E$ values could be due to different testing procedures (e.g., strip extensometry and inflation) and conditions, including different stress levels and application rates, different donor ages, storage and preparation of corneal samples, and the portion of corneal

### Table 1. Hysteresis Values Measured for Two Different Ranges of Application Loads at Various Scan Rates

<table>
<thead>
<tr>
<th>Loads ($\mu N$)</th>
<th>3–9</th>
<th>10–19</th>
<th>20–29</th>
<th>30–39</th>
<th>&gt;40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornea 1</td>
<td>1 ± 0.1*</td>
<td>0.193 ± 0.01</td>
<td>0.210 ± 0.01</td>
<td>0.227 ± 0.02</td>
<td>0.234 ± 0.02</td>
</tr>
<tr>
<td>Cornea 2*</td>
<td>2 ± 0.1*</td>
<td>0.592 ± 0.01</td>
<td>0.602 ± 0.02</td>
<td>0.622 ± 0.01</td>
<td>0.638 ± 0.03</td>
</tr>
<tr>
<td>Cornea 3*</td>
<td>1 ± 0.1*</td>
<td>0.198 ± 0.01</td>
<td>0.199 ± 0.02</td>
<td>0.198 ± 0.00</td>
<td>0.199 ± 0.00</td>
</tr>
<tr>
<td>Cornea 4*</td>
<td>2 ± 0.1*</td>
<td>0.489 ± 0.01</td>
<td>0.573 ± 0.02</td>
<td>0.564 ± 0.01</td>
<td>0.550 ± 0.02</td>
</tr>
<tr>
<td>Cornea 3*</td>
<td>1 ± 0.1*</td>
<td>0.237 ± 0.03</td>
<td>0.284 ± 0.03</td>
<td>0.286 ± 0.03</td>
<td>0.292 ± 0.03</td>
</tr>
<tr>
<td>Cornea 4*</td>
<td>2 ± 0.1*</td>
<td>0.669 ± 0.06</td>
<td>0.748 ± 0.05</td>
<td>0.756 ± 0.03</td>
<td>0.642 ± 0.02</td>
</tr>
<tr>
<td>Cornea 4*</td>
<td>1 ± 0.1*</td>
<td>0.147 ± 0.03</td>
<td>0.154 ± 0.01</td>
<td>0.159 ± 0.00</td>
<td>0.189 ± 0.01</td>
</tr>
<tr>
<td>Cornea 4*</td>
<td>2 ± 0.1*</td>
<td>0.536 ± 0.05</td>
<td>0.589 ± 0.03</td>
<td>0.627 ± 0.03</td>
<td>0.548 ± 0.03</td>
</tr>
</tbody>
</table>

Hysteresis values are expressed in mean picojoules ± SD. * ANOVA: $P < 0.001$ between hysteresis values at different scan rates in each corneal sample.

With slow loading, the specimens recovered more from their deformations and, as a result, they experienced less hysteresis than with fast loading. On the other hand, at microscale level, hysteresis also showed a decrease at the fastest application rates ($>40 \mu m/s$), indicating a nonlinear adaptation of the anterior stromal microstructure in relation to the speed of the locally applied deformation, probably because of reduced fluid movement and stretching between collagen fibers.
in our study, $E_{(local)}$ reflected only the stiffness of the most anterior stroma, probably because of the contribution of the most anterior collagen lamella. Therefore, we cannot compare our results to those from the literature where $E_{(bulk)}$ should be considered a convolution of the properties of all the corneal layers: A nonuniform distribution of elasticity with depth has been demonstrated.\textsuperscript{48} Investigation of the biomechanical response of different corneal layers by AFM could add valuable information to understand the depth-dependent behavior of the tissue at microscale level.\textsuperscript{2,6,9,10}

Previous authors\textsuperscript{49} have characterized, by AFM nanoin- dention, the anterior stromal basement membrane’s modulus of elasticity. Force curves were taken using a $1 \mu m$ radius spherical tip at a $2 \mu m/s$ rate working at an elastic regimen. The mean $E$ value of the anterior basement membrane was $7.5 \pm 4.2$ kPa, and the maximum indentation depth was less than $0.2 \mu m$. This information, together with that provided by our experiment, in which the maximum AFM indentation was at least $1.7 \mu m$ deeper than that achieved by Last et al.\textsuperscript{49} and $E$ values ranged between 1.1 and 2.6 MPa, implies that Bowman’s layer (thickness, $<0.5 \mu m$) does not contribute significantly to mechanical strength within the anterior stroma.\textsuperscript{8} Bowman’s layer is considered a condensation of the superficial layers of the stroma, where collagen fibrils are tightly interlaced and smaller than the underlying layers to support the continuous migration of epithelial basal cells.\textsuperscript{49–51} The roughness measurements of the anterior stromal surface were consistent with AFM data obtained in monkey and porcine corneas.\textsuperscript{52,53} At AFM imaging, the Bowman’s layer showed a feltlike morphology: These features were similar to those observed with scanning electron microscopy.\textsuperscript{49,50}

Although the technique has been well established in biological applications, mechanical testing using the AFM should consider uncertainties in accuracy of the data-processing method because of the assumptions in contact mechanics modeling.\textsuperscript{21,22,44,54,55} The force-depth relationship in any soft material is nonlinear, and meticulous attention should be paid to reliably assess the intrinsic response of the sample. Investi-
gators have attributed nonlinearity of the indentation response entirely to the tip geometry.22,54,55 Contact mechanics models demonstrated to be, in general, fairly accurate in soft biological matters though the fundamental assumptions of the theory are that the sample is a homogeneous, isotropic, linear elastic half-space subject to infinitesimally small strains. In this work, we calculated the modulus of elasticity of the anterior stroma by fitting the Hertz-Sneddon model to force curve data. To indent the anterior human corneal stroma, we adopted an experimental protocol based on the literature16–22,49,54–56 and our previous experience in using AFM in vision science.23,24,55,57 Calibration of both the cantilever elastic constant and the cantilever deflection was performed before and after mechanical testing on tissues; maximum indentation was less than 0.007% of the central stromal thickness; measurements were performed in liquid with 15% dextran solution to maintain the tissue hydration constant; and relatively stiff cantilevers (>20 N/m) with a sharp tip were used to perform microindentation of the tissue beyond Bowman' layer (>500 nm depth) with high-reproducibility (SD <10%). Obtaining reproducible data is fundamental in any mechanical characterization system, to verify the reliability of results. The anterior stromal surface was very smooth, in accordance with reports in previous studies,52,55 further indicating that no damage was caused to the stromal surface by the AFM tip.10 Moreover, damage to the stromal surface could have seen as a discontinuity in the force curve during AFM force spectroscopy.56 Only the central region of the anterior stroma was analyzed to minimize possible variations due to the meridional-dependent mechanical anisotropy of the tissue and artifacts that may have been induced by forces manipulation at the edges of the tissues.3,5,7

AFM investigation of donor donor corneas with differences in age or clinical diagnosis of keratoconus would add valuable information to our clinical understanding of the micromechanical behavior of the human stroma. A thorough description of the local properties of the human cornea at micrometer level could enhance our understanding of the tissue’s biomechanics and ultimately be valuable in optimizing the design and development of bioengineered corneas. Efforts in modeling the biomechanics of the human corneal tissue at micrometer level are needed.

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