Normal Human Keratocyte Density and Corneal Thickness Measurement by Using Confocal Microscopy In Vivo

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Purpose. To quantify keratocyte density according to stromal region and subject age and to measure the thickness of the normal human cornea and its layers in vivo.

Methods. Seventy normal corneas of 70 subjects were examined by confocal microscopy (contact lens wearers were excluded). Ages of subjects ranged from 12 to 80 years, with 10 subjects per decade. Images were recorded by continuously focusing the optical section through the full-thickness central cornea. The independent human observers manually identified bright objects (keratocyte nuclei) against a dark background to quantify keratocyte density. This method was validated histologically in three human corneas. Thickness measurements were obtained by plotting mean reflected light intensity in images against corneal depth, and calculating distances between intensity peaks that corresponded to corneal layers.

Results. Full-thickness central keratocyte density was 20,522 ± 2,981 cells/mm² (mean ± SD, n = 69). The number of keratocytes in a full-thickness column of central stroma, which had a cross-sectional area of 1 mm², was 9624 ± 1385 cells. Keratocyte density was highest in the anterior 10% of the stroma. Full-thickness keratocyte density was correlated with age (r = −0.62, P < 0.001), decreasing 0.45% per year. Central corneal thickness was 563.0 ± 31.1 μm (mean ± SD) and central epithelial thickness was 48.6 ± 5.1 μm.

Conclusions. This is the first study to quantify regional keratocyte density comprehensively in vivo across a broad age range of normal human subjects. The method was acceptable to both subject and observer, and may prove useful for quantifying keratocyte density in patients with corneal disorders or after corneal surgery. (Invest Ophthalmol Vis Sci. 2001;42:333–339)

Keratocytes are fibroblast-like cells in the corneal stroma responsible for maintaining the integrity of this layer of the cornea. The ability to measure keratocyte density would be valuable in studying the role of these cells in familial stromal wound healing, for example, after refractive surgery. Keratocyte de-polymerization due to apoptosis after epithelial scraping is postulated to be the stimulus for stromal regeneration after phototherapeutic keratectomy (PRK), and the clinical haze seen in the early months after PRK may in part be due to an increased density of keratocytes. Human keratocyte density has been estimated indirectly by measuring stromal DNA content. Although, this method has been used to assess variation in normal human keratocyte density with stromal region and with age, it is invasive and cannot be used to study keratocyte density in vivo.

Confocal microscopy in vivo has been used to study the cornea at the cellular level to describe normal morphology, keratitis, other pathologic conditions, and also the effects of refractive surgery. The intensity profile generated in vivo from reflected light in confocal images has been used to obtain total corneal and regional thickness measurements in normal and PRK-treated corneas. The amount of light reflected from PRK-treated corneas has been measured and correlated with subjective assessment of clinical haze. Modulating effects of topical agents on haze after PRK have been assessed using this method. The densities of normal corneal cell populations, including keratocytes, have also been measured from confocal microscopy images in vivo, and changes in keratocyte density have been quantified after PRK. In the latter studies, manual counting methods were used on a limited number of images. Automated quantification of keratocyte density in humans has been attempted, but these methods have not been validated histologically.

In the present study, we used confocal microscopy in vivo to quantify keratocyte density in normal human corneas across a broad age range of subjects. We assessed keratocyte density in vivo in the central cornea and its variation with depth of stroma.

Methods

Subjects

Seventy corneas of 70 subjects were studied. None had any history of contact lens wear, anterior segment disease, ocular trauma or surgery, diabetes mellitus or the use of ocular medications. Systemic medications were permitted unless they were known to affect the cornea or anterior segment. Each cornea was examined by slit lamp biomicroscopy to ascertain that it was normal. Intraocular pressures were measured by applanation tonometry. Subjects were assigned to seven subgroups corresponding to the seven decades of life from 11 to 80 years. Ten volunteers, five of each sex, were recruited to each subgroup. Mean age was ±5.9 years with an age range from 12 to 80 years. Ten subjects were Asian, one was Hispanic, and the remainder were white. This study was approved by our Institutional Review Board and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all adults and the parents of minors after explanation of the nature and possible consequences of the study.

Confocal Microscopy In Vivo

A tandem scanning confocal microscope (Tandem Scanning, Reston, VA) was used to examine corneas in vivo. The microscope had a ×24, 0.6 numeric aperture objective lens with a concave surface and a working distance of 0 to 1.5 mm. The position of the optical section could be advanced or retracted by an internal lens without changing the position of the front surface of the objective. This was controlled from a computer joystick, custom-mounted onto the mechanical joystick of the microscope, and connected to an encoder mike controller (Oriel 18011; Oriel Instruments, Stratford, CT) through a computer.
workstation (INDY; Silicon Graphics, Mountain View, CA). The mounted computer joystick was also used to begin image acquisition with continuous advancement of the focal plane. Images were digitized directly from a low-light camera (VE-1000 ST; Dage-MTI, Michigan City, IN) and were stored in computer memory.

The microscope had been calibrated as outlined in a previous study. Proparacaine hydrochloride 0.5% (Bausch & Lomb Pharmaceuticals, Tampa, FL) was instilled into the eye to be examined. The objective lens was disinfected by using 70% isopropyl alcohol wipes before and after each examination. A drop of 2.5% hydroxypropyl methylcellulose (CIBAVision Ophthalmics, Atlanta, GA) optical coupling medium was placed on the tip of the objective lens, and the lens was manually advanced until the medium contacted the central cornea. A series of confocal images (which constituted one “scan”) was recorded as the focal plane was advanced from anterior to the epithelium to posterior to the endothelium (continuous through-focusing). Images were digitized during each scan by the workstation and stored in its memory at 30 frames/sec. Each image represented a coronal section approximately 475 × 350 μm (horizontal × vertical).

The average z-depth separation (Δz) between optical centers of adjacent images was 2.6 μm, the thickness of the human cornea in vivo in 22 subjects measured by an ultrasonic pachometer (model 1000; DGH Technology, Frazer, PA) divided by the number of through-focus confocal images between the anterior and posterior surfaces in those subjects (2.60 ± 0.07 μm, mean ± SD, n = 22). The average speed of advancement of the focal plane, therefore, was 78 μm/sec.

Scans were obtained four to eight times from the cornea of one eye per subject. The objective was withdrawn from the cornea after each scan. Images were acquired by using two camera modes: a fixed-gain mode, in which the camera was set at a constant gain, voltage, and black level, and an automatic-gain mode, in which these parameters were automatically adjusted by the camera throughout image acquisition. This method of confocal image acquisition is a direct extension of the method presented in our previous study. Although image acquisition required 10 to 15 seconds per scan, the contact time between objective and cornea was 15 to 40 seconds per scan, depending on the ability of the subject to cooperate with confocal examination.

Keratocyte Density Measurement
Keratocyte density was measured from one confocal scan per subject, from images acquired in the automatic-gain camera mode. All scans were reviewed, and the scan with the least lateral ocular movement (motion blur) and with no anteroposterior movement of the cornea relative to the objective was selected for analysis. Two independent human observers selected two images from each of five anteroposterior regions of stroma in which keratocyte density was to be measured. The images selected contained no motion blur, as determined by the appearance of well-defined borders of objects in the images (Fig. 1), and were selected from times during the scan when the globe was stationary.

Two independent human observers manually counted bright objects (keratocyte nuclei) in the selected images (Fig. 1). The digital images were loaded into an interactive computer program and displayed in random order. A counting area was defined for each image by superimposing a box boundary; the same boundary was used for all images. By convention, objects overlapping the edges of the boundary were only counted on two sides of the box (left and lower sides). Each object representing a single keratocyte nucleus was marked once by using the interactive computer display. The number of keratocyte nuclei represented by a large object was judged by the observer according to the area of the large object relative to the area of objects representing single keratocyte nuclei in the same image. Large objects were marked as many times as the number of keratocyte nuclei they were judged to represent. The number of marks placed in each image was counted by the computer, and keratocyte density was expressed as cells per unit volume for each image by using the stereologic methods outlined in our previous paper. We used the mean of the densities measured by the two observers for each image as the keratocyte density for that image.

Histologic Validation
After confocal microscopy in vivo, three corneas of three subjects were retrieved for histologic examination (eyes were enucleated because of choroidal melanoma). Full-thickness keratocyte density was manually measured by two observers by counting stained nuclei in 10 histologic sections of each central cornea. This density was compared with full-thickness keratocyte density measured by confocal microscopy in vivo. Tissue preparation, correction for shrinkage, cell counting, and stereologic methods were used to estimate cell density, as described previously.

Corneal Thickness Measurements
We wrote software that plotted an intensity profile of reflected light from confocal images obtained by continuous through-focusing of the focal plane, as described by Li et al. Images were acquired with the camera operating in its fixed-gain mode and were taken from the central and temporal (2.5 mm from the limbus) cornea. Intensity was estimated from the mean grayscale value in a 300 × 300-pixel area in the center of each image. Peaks in intensity corresponded to the superficial epithelium, the endothelium, and often the subbasal nerve plexus and the most anterior keratocytes (Fig. 2). Corneal thickness was the distance between superficial epithelial and endothelial peaks. Epithelial thickness was the distance between the superficial epithelial peak and the subbasal nerve plexus peak (basal aspect of the basal

FIGURE 1. Confocal microscopy in vivo image from the cornea of a 37-year-old subject. Keratocyte nuclei appeared as well-defined bright objects against a dark background in images without motion blur (left). Two independent observers manually marked keratocyte nuclei in selected images by using an interactive computer program (right) to calculate keratocyte density. By convention, objects overlapping the edges of the box boundary were counted on only two sides of the box, the left and lower sides. Bar, 100 μm.
epithelial cells\textsuperscript{29,30}; Bowman’s layer thickness was the distance between the subbasal nerve plexus peak and the peak corresponding to the most anterior keratocytes; and stromal thickness was the distance between the peak corresponding to the most anterior keratocytes and the endothelial peak and therefore included Descemet’s membrane. If intensity peaks corresponding to the subbasal nerve plexus or the most anterior keratocytes did not appear, the first focused image of each was manually defined and used to determine thickness. Intensity profiles were also generated from the images obtained with the camera operating in the automatic-gain mode. Under this condition, intensity peaks corresponded to the superficial epithelium and the endothelium.

Movement of the optical section is not linear but is a cubic polynomial of the video image number from the surface of the objective lens, according to the specifications of the manufacturer. In this study, the image of the objective was recorded in 47% of fixed-gain central scans (32/68) and temporal scans (29/62). Thickness data for each of these scans were calculated with the polynomial equation and knowledge of the position of the cornea relative to the objective. For the 53% of scans that did not contain the image of the objective, we calculated thickness with the polynomial equation and the position of the cornea relative to the first image in the scan.

Central corneal thickness was also measured by calculating the mean of three ultrasonic pachometry recordings in 22 subjects.

Data Analysis

We first generated a reflected light intensity profile to define the range of images of stroma (termed full-thickness stroma) from which keratocyte density would be measured. The first image in the range was the first focused image of keratocyte nuclei, and thus Bowman’s layer was not included. The last image in the range was defined as having a reflected light intensity similar to other images of stroma but without endothelial cells, in an attempt to exclude Descemet’s membrane. The full-thickness stroma was divided into anterior, middle, and posterior thirds, and the anterior and posterior thirds were further divided into two unequal regions such that the anterior and posterior 10% of the full-thickness stroma, respectively, were represented. The mean of the two images selected from each of the five anteroposterior regions was used as keratocyte density for each region. In the anterior 10% of the stroma, one of the two selected images was the most anterior countable image. The z-depths of the selected images were known, and the position of each image within the stroma was expressed as a percentage of the full-thickness stroma.

We used the keratocyte densities of all 10 selected images to calculate keratocyte density for the full-thickness stroma for each subject. We first calculated the absolute number of keratocytes in a full-thickness column of central stroma, which had a cross-sectional area of 1 mm\textsuperscript{2}. Theoretically, this is the area under the curve in a plot of central keratocyte density versus central stromal thickness. Because we measured keratocyte density at only 10 z-depths within the stroma, located at irregular intervals, we approximated the area under the curve by assuming keratocyte density at each measured z-depth represented the mean keratocyte density over a thickness of stroma superficial and deep to that z-depth. This thickness varied depending on the distances between adjacent selected images and was equal to the sum of the half-distances between the image in question and each of the two adjacent selected images. We calculated full-thickness keratocyte density by dividing the absolute number of keratocytes by the central stromal thickness.

Variations in keratocyte density with depth of stroma were demonstrated by generating a profile of keratocyte density measured from each selected image of each subject according to the position of the image within the stroma. Mean keratocyte density was also calculated for each anteroposterior region and for the most anterior countable image. Significance of differences between adjacent depths of stroma were determined by using paired Student’s t-tests. Comparisons were adjusted using the Bonferroni technique. Adjustment was completed for the four adjacent comparisons among the five anteroposterior regions. Correlations between keratocyte density and age and between keratocyte number and age were assessed using Spearman’s rank correlation ($r_s$) for non-normal data. We computed the annual rate of decrease in full-thickness keratocyte density and keratocyte number by
RESULTS

Histologic Validation

Full-thickness central keratocyte densities measured by confocal (22.050 ± 3.243 cells/mm³, mean ± SD) and histologic (23.120 ± 3.717 cells/mm³) methods did not differ (P = 0.10, n = 3). The minimum detectable difference between full-thickness keratocyte densities measured by the two methods was 1810 cells/mm³ (paired analysis, n = 3, α = 0.05, β = 0.20).

Keratocyte Density

All 70 subjects tolerated confocal microscopy but images from one subject contained too much motion blur for keratocyte density measurement and were therefore excluded. Although we attempted to count 10 images from each subject, this was not always possible if images were blurred by motion. We counted keratocytes in 10 images in 57 subjects, 9 images in 9 subjects, 8 images in 2 subjects, and 7 images in one subject; cells were counted in a total of 674 images. The difference between densities determined by the two observers was 13 ± 3710 cells/mm³ (mean ± SD, n = 674) and this did not differ from zero (P = 0.37, Wilcoxon signed-rank test). The minimum detectable difference between densities by the two observers was 400 cells/mm³ (paired analysis, α = 0.05, β = 0.20, n = 674).

Full-thickness central keratocyte density was 20.522 ± 2.981 cells/mm³ (mean ± SD, n = 69). The number of keratocytes in a full-thickness column of central stroma, which had a cross-sectional area of 1 mm², was 9624 ± 1385 cells. Central keratocyte densities of all images from all subjects are shown with distance through the stroma in Figure 3. In general, keratocyte density was highest in images adjacent to Bowman’s layer and decreased through the first 10% of the stroma. Keratocyte density measured from the most anterior countable image of each subject was 33,050 ± 11,506 cells/mm³ (Table 1). The z-depth of the most anterior countable image was 2.0% ± 1.9% of stromal thickness from the most anterior keratocytes (mean ± SD, range 0%-8.0%). Keratocyte density decreased slightly through the remaining depth of the stroma. In the posterior 10% of the stroma (pre-Descemet region), keratocyte density was not significantly higher than it was in the remainder of the posterior third of the stroma (adjusted P = 0.16). The Bonferroni-adjusted minimum detectable difference between densities for these two regions was 1211 cells/mm³ (paired analysis, n = 67, adjusted α = 0.05/4 = 0.0125, β = 0.20; Table 1).

Full-thickness keratocyte density did not differ between males (51% of subjects) and females (P = 0.74, unpaired Student’s t-test) or between right (57% of eyes) and left eyes (P = 0.31, unpaired Student’s t-test).

Full-thickness central keratocyte density was negatively correlated with age (Fig. 4, Table 2) and decreased 0.45% per year. Keratocyte densities in all anteroposterior regions were negatively correlated with age except the posterior 67% to 90% region of the stroma (Table 2). The number of keratocytes in the full-thickness stroma was also negatively correlated with age (rc = −0.58, P < 0.001, n = 69) and decreased 0.43% per year (Fig. 5).

Corneal and Regional Thickness

Through-focus scans in the fixed-gain camera mode were obtained in 68 subjects for the central cornea and in 64 subjects for the temporal cornea, although two in the latter group were excluded. Distance through the stroma was determined by the image thickness keratocyte densities measured by the two methods. Distance through the stroma was assessed by calculating the mean z-depth of cell loss per year and expressing the result as a percentage.

The thickness of the cornea, epithelium, Bowman’s layer, and remaining stroma (with Descemet’s membrane) were measured in the central and temporal cornea from one scan acquired with the camera operating in the fixed-gain mode. The presence or absence of the nerve plexus was noted. Central and temporal corneal thicknesses were also measured from scans acquired in the automatic-gain camera mode. Agreement between corneal thickness measurements from images acquired in both camera modes was assessed by calculating the mean difference and the SD of the differences. We assessed the Spearman rank correlations (r_s) between age and corneal, epithelial, and stromal thickness and the Pearson correlation coefficients (r_p) between full-thickness keratocyte density and corneal and stromal thickness, and between keratocyte number and corneal and stromal thickness. Central corneal thicknesses measured by ultrasonic pachometry and confocal microscopy were compared by using a paired Student’s t-test.

Table 1. Normal Central Human Keratocyte Density by Anteroposterior Stromal Region

<table>
<thead>
<tr>
<th>Stromal Depth (% Stromal Thickness)</th>
<th>Keratocyte Density</th>
<th>P*</th>
<th>MDD†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-thickness stroma</td>
<td>20,522 ± 2,981</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Most anterior countable image‡</td>
<td>33,050 ± 11,506</td>
<td>&lt;0.001</td>
<td>--</td>
</tr>
<tr>
<td>0-10% (anterior)</td>
<td>28,838 ± 8,913</td>
<td>&lt;0.001</td>
<td>--</td>
</tr>
<tr>
<td>11-33%</td>
<td>20,916 ± 4,032</td>
<td>&lt;0.001</td>
<td>--</td>
</tr>
<tr>
<td>34-66% (mid)</td>
<td>19,241 ± 2,906</td>
<td>1.0</td>
<td>1,220</td>
</tr>
<tr>
<td>67-90%</td>
<td>19,081 ± 2,703§</td>
<td>0.16</td>
<td>1.211</td>
</tr>
<tr>
<td>91-100% (posterior)</td>
<td>19,947 ± 3,254</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Keratocyte density is expressed in mean cells per cubic millimeter ± SD (n = 69).

* Bonferroni-adjusted paired Student’s t-tests comparing keratocyte density with the next deeper stromal region (adjusted P = unadjusted P × 4).

† Bonferroni-adjusted minimum detectable differences (MDD) in keratocyte density (in cells per cubic millimeter) compared with the next deeper stromal region (paired analysis, adjusted α = 0.05/4 = 0.0125, β = 0.20).

‡ The most anterior countable image was at z-depth 2.0% ± 1.9% of stromal thickness from the most anterior keratocytes (mean ± SD, range 0%-8%).

§ n = 68; data were not analyzed due to motion blur.

|| n = 67; data were not analyzed due to motion blur.
excluded because the subjects blinked. Central and temporal corneal thicknesses were 563.0 ± 31.1 μm and 651.4 ± 57.3 μm, respectively (mean ± SD, Table 3). Central and temporal epithelial thicknesses were 48.6 ± 5.1 μm and 51.0 ± 8.7 μm, respectively, and did not differ (P = 0.08; Table 3). In the 47% (61/130) of scans that included the surface of the objective lens, the distance between the objective and the epithelium was 76.5 ± 22.8 μm (range, 41.1–125.2 μm).

The subbasal nerve plexus was visible in the central cornea of 81% of subjects (55/68). In a further 13% (9/68) of subjects, there was a peak in the intensity profile, but no nerve plexus was seen (Table 3). In the temporal cornea, the subbasal nerve plexus was visible in only 37% (23/62) of subjects, and a peak with no corresponding nerve plexus was noted in 21% (13/62) of subjects (Fig. 2, Table 3).

There was no significant correlation between age and corneal thickness centrally (r = 0.04, P = 0.75, n = 68) and temporally (r = -0.16, P = 0.20, n = 62) or between age and epithelial thickness centrally (r = -0.06, P = 0.65, n = 55) and temporally (r = -0.35, P = 0.13, n = 23). There was also no significant correlation between age and central stromal thickness (r = -0.08, P = 0.52, n = 67). Full-thickness keratocyte density was significantly correlated with central corneal thickness (r = -0.31, P = 0.01, n = 67) and central stromal thickness (r = -0.28, P = 0.02, n = 67; Fig. 6). The number of keratocytes in the full-thickness central stroma did not correlate with central corneal thickness (r = 0.10, P = 0.43, n = 67) or central stromal thickness (r = 0.13, P = 0.28, n = 67).

**Table 2.** Correlation between Central Anteroposterior Keratocyte Density and Age

<table>
<thead>
<tr>
<th>Stromal Depth (%)</th>
<th>Correlation and Significance (n = 69)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-thickness stroma</td>
<td>r_s = -0.62, P &lt; 0.001</td>
</tr>
<tr>
<td>0–10% (anterior)</td>
<td>r_s = -0.50, P &lt; 0.001</td>
</tr>
<tr>
<td>11–53%</td>
<td>r_s = -0.71, P &lt; 0.001</td>
</tr>
<tr>
<td>34–66% (mid)</td>
<td>r_s = -0.50, P &lt; 0.001</td>
</tr>
<tr>
<td>67–90%</td>
<td>r_s = μ = 0.12, P = 0.32*</td>
</tr>
<tr>
<td>91–100% (posterior)</td>
<td>r_s = -0.35, P = 0.005†</td>
</tr>
</tbody>
</table>

* n = 68; data were not analyzed due to motion blur.
† n = 67; data were not analyzed due to motion blur.

**DISCUSSION**

We have successfully used confocal microscopy to determine human keratocyte density in vivo as a function of corneal stromal region and subject age, and the method was shown to be valid and acceptable.

Full-thickness central keratocyte density in our study was 20,522 cells/mm² (mean age, 46 years). This was higher than the 16,000 cells/mm² (47 corneas of 25 subjects, ages 25–56) estimated by Stave et al., although it was lower than the 41,000 cells/mm² (mean age, 77 years) measured by Möller-Pedersen et al. Prydal et al. used an automated method to estimate keratocyte density as cells per unit area from confocal images in four subjects and found a mean two-dimensional density of 265 cells/mm². The mean two-dimensional density of cells in our images before conversion to a volumetric density was 328 cells/mm², somewhat higher than the two-dimensional density found by Prydal et al. We showed keratocyte density was highest in the anterior stroma of the central cornea in agreement with previous human, and porcine studies.

We demonstrated a decrease in full-thickness central keratocyte density with age by 0.45% per year. Although keratocyte density was negatively correlated with stromal thickness, we showed that the absolute number of keratocytes in the full-thickness stroma also decreased with age by 0.43% per year. Möller-Pedersen noted a similar correlation between keratocyte density and age in the central (7 mm diameter) cornea with keratocyte loss of 0.5% per year of life. We also showed that all anteroposterior regional keratocyte densities were neg-
Table 3. Thickness Measurements from the Reflected-Light Intensity Profile

<table>
<thead>
<tr>
<th>Layer of Cornea Measured</th>
<th>Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>Temporal</td>
</tr>
<tr>
<td>Superficial epithelium to endothelium (corneal thickness)</td>
<td>563.0 ± 31.1 ($n = 68$)</td>
</tr>
<tr>
<td>Superficial epithelium to nerve plexus (epithelial thickness)</td>
<td>48.6 ± 5.1 ($n = 55$)</td>
</tr>
<tr>
<td>Nerve plexus to most anterior keratocytes (Bowman’s layer thickness)</td>
<td>16.7 ± 4.4 ($n = 55$)</td>
</tr>
<tr>
<td>Superficial epithelium to peak with no visible nerves</td>
<td>49.0 ± 4.0§ ($n = 9$)</td>
</tr>
<tr>
<td>Peak with no visible nerves to most anterior keratocytes</td>
<td>14.1 ± 5.3∥ ($n = 9$)</td>
</tr>
<tr>
<td>Most anterior keratocytes to endothelium</td>
<td>498.5 ± 29.4 ($n = 68$)</td>
</tr>
</tbody>
</table>

Thickness data are expressed in micrometers.

* Paired Student’s t-tests (unless stated) comparing central and temporal cornea.
† Minimum detectable difference (MDD; in micrometers) between central and temporal cornea (paired analysis, $\alpha = 0.05$, $\beta = 0.20$).
‡ Wilcoxon signed-rank test.
§ $P = 0.87$ (central) and $P = 0.19$ (temporal), compared with epithelial thickness (unpaired Student’s t-tests).
∥ $P = 0.21$, compared with central Bowman’s layer thickness (Wilcoxon rank-sum test).
¶ $P = 0.008$, compared with temporal Bowman’s layer thickness (Wilcoxon rank-sum test).

The use of the reflected light intensity profile to measure corneal and sublayer thickness was first described by Li et al. 20
We measured central epithelial thickness of 48.6 μm, lower than the 50.6 μm measured by Li et al., and Bowman’s layer thickness of 16.7 μm, in agreement with the 16.6 μm reported by Li et al. Our data were based on one through-focus scan per subject, whereas Li et al. used several scans. Li et al. corrected their depth measurements for the nonlinear separation of video frames by noting the position of the epithelium before each scan. In our experience, because of eye movements, the epithelial position noted in this manner often changed before the scan was executed. Therefore, we corrected for the positional nonlinearity by counting the number of frames between the image of the objective and the surface of the epithelium.

The precision of distances measured between two surfaces is limited by our ability to specify each surface as being within the scan distance of a particular video image. Consequently, distances estimated from the number of video images between two objects or surfaces can only be specified to within the distance scanned during one video image, or ± 2.6 μm.

The nerve plexus was visible more frequently in images of the central cornea than the peripheral cornea, which may confirm findings by Müller et al. that fewer nerve fibers were encountered in the peripheral than central cornea when examined by electron microscopy. By confocal microscopy,

Table 4. Agreement between Two Repeated Measurements of Corneal Thickness

<table>
<thead>
<tr>
<th>Layer of Cornea Measured</th>
<th>Difference*</th>
<th>MDD†</th>
<th>Difference*</th>
<th>MDD†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>—2.2 ± 17.0 ($P = 0.30$)∥</td>
<td>5.8</td>
<td>8.2 ± 40.0 ($P = 0.11$)∥</td>
<td>14.2</td>
</tr>
<tr>
<td>Temporal</td>
<td>—</td>
<td></td>
<td>—</td>
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</tr>
</tbody>
</table>

Central cornea, $n = 67$; temporal cornea, $n = 62$.

* Mean difference ± SD of the differences (in micrometers) between measurements from images acquired in the fixed-gain and automatic-gain camera modes for each subject (fixed-gain mode — automatic-gain mode).
∥ Minimum detectable difference (MDD; in micrometers) between repeated measurements (paired analysis, $\alpha = 0.05$, $\beta = 0.20$).
‡ Paired Student’s t-tests.
however, only nerve fiber bundles are visualized and not individual nerve fibers. We noted peaks in the intensity profile in the region corresponding to the nerve plexus in 9 of 13 central corneas in which nerves were not seen (Fig. 2, Table 4). Similar peaks in the absence of nerves have been noted in patients 1 week after laser in situ keratomileusis (Patel SV, unpublished data, 1999). This suggests that the intensity peak is not generated entirely from the nerve plexus, but contributions from the basal lamina or Bowman’s layer may also be responsible.

In the present study, eyes were stabilized only by patient self-fixation and the viscous coupling between the objective and the cornea, whereas Stave et al. reduced motion blur by applying a low-vacuum suction cup to stabilize the eye. In our previous study, we examined rabbits under general anesthesia and were able to use a custom automated algorithm to measure keratocyte density. Motion blur was of little significance in that study, but has been a limiting factor when applying the same algorithm to confocal images of moving human corneas. This study has provided comprehensive data of human keratocyte density in vivo in different corneal regions and across a broad age range of normal subjects. The same method may be useful to study pathologic conditions and to estimate changes in keratocyte density after keratorefractive procedures.

References