Ultrastructure in Anterior and Posterior Stroma of Perfused Human and Rabbit Corneas

Relation to Transparency

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Purpose. The authors sought to discover whether there are differences in the degree of spatial order in the fibrillar ultrastructure between anterior and posterior stroma.

Methods. Human corneas were obtained from eye bank eyes. Although they had been classified as normal, some swelling remained after 3 hours of deturgescence. Freshly excised, unswollen rabbit corneas also were used. Image analysis methods were applied to transmission electron micrographs of the anterior, middle, and posterior stroma of these corneas to determine the positions and radii of fibrils, the fraction of total area occupied by fibrils, and the fibril number density. Results were used to calculate the interference factor that appears in the direct summation of the fields for light scattering theory and to estimate the total scattering cross-section per fibril. The interference factor is a measure of the spatial order in the positions and sizes of the fibrils.

Results. Electron micrographs showed anterior–posterior variations in size and number density of fibrils. The interference factor at wavelengths of visible light was lower in posterior stroma than in anterior stroma for humans and rabbits. In some instances in humans, the anterior interference factor was characteristic of mildly swollen cornea. When averaged for the electron micrographs analyzed, the anterior stroma was predicted to scatter approximately twice as much light per unit depth as the posterior stroma in humans (at any given wavelength) and approximately three times as much in rabbits.

Conclusions. Calculations of the interference factor showed that there were differences in the anterior–posterior spatial ordering of fibrils. In human corneas, the differences could have been caused by intrinsic in vivo differences between anterior and posterior stroma; however, possible anterior–posterior variations in swelling between the two regions in vitro also could have affected the results. Invest Ophthalmol Vis Sci. 1995;36:1508–1523.

Several studies have suggested that there are differences in the structure and properties between anterior and posterior regions of the corneal stroma. Polack1 showed that the anterior lamellae of human cornea are interwoven, whereas lamellae in the posterior are not. These findings were confirmed more recently by Smolik and McCarey2 and by Komai and Ushiki.3 Lamellae in rabbit cornea apparently do not interweave. McTigue4 noted that the anterior one third of the stroma appears to have a different developmental origin from the posterior two thirds, and he postulated that this accounts for some of the structural differences observed in the lamellae within these regions. Studies on both rabbit and bovine cornea have shown that the posterior stroma is more hydrated5 and has a greater capacity to swell than the anterior stroma.6,7 It has been shown also that human and rabbit corneas swell more rapidly when the swelling is induced from the posterior surface.8 Finally, experiments on bovine cornea show that the concentration and types of glycosaminoglycans also vary with corneal depth.9,10 Differences between anterior and posterior stromal ultra-
structure is of potential importance in understanding the effects of photokeratectomy.

Glycosaminoglycans are thought to play an important role both in the interactions between collagen fibrils and in corneal hydration, thus, variations in their concentrations in the anterior and posterior cornea provide possible mechanisms for differences in the stromal ultrastructure between the two regions. Stromal light scattering depends sensitively on the fibrillar ultrastructure, especially on the degree of order in the spacing of fibril centers and, to a lesser degree, on fibril size. We have developed methods for calculating light scattering from the fibril arrangements depicted in transmission electron micrographs (EMs) and have shown that they agree closely with scattering deduced from transparency measurements made on fresh (unfixed) tissue. Various methods have been developed to indicate how the fibrillar ultrastructure would affect light scattering, and the qualitative features of these techniques are reviewed in the remaining paragraphs of this section.

Theories of corneal transparency seek to explain the almost complete absence of light scattering in the stroma in the presence of the multitude of collagen fibrils of which it is composed. All the theories model the stroma as a single lamella made of parallel fibrils, with the lamella’s thickness that of the entire cornea. This model can be justified for analysis of transparency or total scattering cross-section, but not for angular scattering calculations. Historically, the fibrils have been treated as thin, infinitely long, dielectric cylinders, each having the same diameter, the same refractive index, and the same perpendicular orientation with respect to the propagation direction of the incident (unpolarized) light.

Normal corneas are transparent to the visible light spectrum as a result of three factors. First, the fibrils, which are the main (nonspecular) scattering elements in the cornea, are weak scatterers because their radii are much smaller than the wavelength of light and their index of refraction is nearly equal to that of the surrounding ground substance. Second, the cornea is thin. This is significant because the amount of scattering is directly proportional to the number of scatterers encountered by the incident beam passing through the cornea. Third, interference effects among waves scattered by different fibrils reduce the amount of scattering from that which would occur if the fibrils within the stroma were scattered independently of one another. The interference factor, which shows how much the scattering is reduced, depends on the spatial distribution of fibril axes and, to a lesser extent, on the spatial distribution of scatterer strength. It is this interference factor that will be used as a measure of order in this article.

The assumption that fibrils scatter independently corresponds to the assumption that fibril positions are uncorrelated and thus are distributed randomly with ideal gas-like disorder. In that case, the interference factor would be unity, and, for typical values of fibril parameters, the cornea would not transmit enough light for normal vision to occur. Consequently, most modern theories of transparency for normal corneas are based on the assumption that the fibrils possess some amount of spatial ordering. Spatial ordering produces destructive interference that lowers the interference factor and reduces the total amount of scattered light in all directions except the forward direction, leading to increased transparency. Calculations based on the structures depicted in EMs indicate that destructive interference reduces the total scattering cross-section by a factor of approximately 10. We remark here that, although the interference factor is an important element in explaining transparency, the other two factors cannot be ignored and they will dominate in some regions of the cornea. For example, the fibrils within Bowman’s layer are highly disordered and scatter light independently of one another, indicating an interference factor of 1. However, Bowman’s layer is transparent in spite of the lack of any interference effects. This is primarily because Bowman’s layer is thin (approximately 12 μm), which is approximately 40 times thinner than the stroma. In addition, the fibrils within Bowman’s layer are roughly two thirds the diameter of those in the stroma. It is reasonable to assume that the refractive indices of the ground substance and the fibrils within Bowman’s layer are essentially the same as those of the stroma.

One difference among the various transparency theories is the type of spatial order assumed for the fibrils within a given lamella. Theories based on long-range order, in which the fibril positions are correlated over long (infinite) distances, assume either that the fibrils are positioned on ideal crystal lattice sites or that they are perturbed randomly a small distance from ideal lattice sites. For fibrils on ideal crystal lattice positions, analysis shows that the interference factor is zero and that such a stroma would be perfectly transparent. With perturbations from the ideal lattice positions that are isotropic and small compared to the light wavelength, a Taylor series expansion can be performed and shows that the interference factor is proportional to the mean square displacement divided by the square of the wavelength.

Theories based on short-range order, as seen in EM, are based on the premise that fibrils in lamella are spatially ordered up to a correlation distance comparable to a few fibril diameters, but beyond that point

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In analogy with x-ray scattering from liquids, the interference factor for light scattering from such an assembly of fibrils can be expressed in terms of an integral whose integrand contains the radial distribution function and a factor that depends on the light wavelength. In the long-wavelength limit, the interference factor reduces to the mean squared fluctuation in the number of fibrils within a region larger than the correlation area normalized by the mean number of fibrils in such a region. The radial distribution function, denoted by \( g(r) \), is the ratio of the local number density of fibril centers at a distance, \( r \), from the axis of a reference fibril to the average number density of fibril centers, \( \rho \). Therefore, it represents the relative likelihood of finding two fibril centers separated by a distance, \( r \). Because two fibrils cannot approach each other closer than touching, the function is zero from \( r \) equal to zero out to a distance at least twice the fibril radius. For cornea, the function is zero beyond this distance of closest approach, and it rises to its first maximum at the most probable separation distance. Beyond the distance over which the fibril centers are correlated, \( g(r) \) becomes unity. The radial distribution function, therefore, also provides a measure of the distance over which fibrils are ordered; however, the interference factor obtained from the integral provides a better measure of the degree to which the fibrils are ordered. As noted above, calculations based on analyses of the ultrastructures in EMs suggest that the interference factor is approximately one tenth for visible light.

The theories discussed to this point are applicable only to fibrils that are of equal size and have a uniform or radially symmetric spatial distribution. The direct summation of fields method is a statistical technique that generalizes these theories to assemblies of fibrils with an arbitrary distribution of diameters (that is, fibril scattering strengths) and with an arbitrary spatial distribution. This technique is expected to be especially beneficial for scarred corneas that show large variability in fibril diameters and for swollen corneas that have voids in the spatial distribution of fibril axes. The method will be described in some detail under Theory in the Methods section. Previous analyses of EM of normal corneas showed a reasonably tight distribution of fibril diameters, with variability on the order of 10%, and no apparent spatial inhomogeneities. However, rather than prejudge the significance of the small variations in fibril diameters or the homogeneity of the spatial distribution, the direct summation of fields method was combined with recently developed image processing methods to analyze EM of the anterior, middle, and posterior stromal regions of endothelial perfused normal rabbit corneas and endothelial perfused human eye bank corneas. The individual fibril positions and radii, fibril number density, and area fraction occupied by fibrils were obtained in the different regions. These data were used in the direct summation of fields method to calculate how the degree of spatial ordering in the fibril positions and scatterer strengths (as measured by the interference factor) and light scattering differ across the stroma in the two species. It should be stressed that no assumptions were made regarding the homogeneity of the fibril distribution so the presence of any inhomogeneities should be reflected in the calculated interference factor.

**METHODS**

**Perfusion of Corneas**

Human eyes were obtained from the Georgia Eye Bank. Their corneas, which were highly transparent and were classified as normal, were excised and the epithelium was removed. They were then mounted under an in vitro specular microscope, and their anterior surfaces were bathed in silicon oil, as described by Dikstein and Maurice, in which they were degassed by perfusing the endothelium with BSS Plus (Alcon Surgical, Ft. Worth, TX) at 37°C. The perfusion rate was 100 μl/minute, and a hydrostatic pressure difference between 15 and 18 mm Hg was maintained during the entire process. Central corneal thickness was measured at the beginning of perfusion and subsequently was measured at 15-minute intervals for the entire 3-hour perfusion time. The central deep epithelialized corneal thicknesses after endothelial perfusion were 0.565 mm, 0.638 mm, 0.662 mm, and 0.570 mm for corneas HE246, HE194, HE201, and HE245, respectively. Although these corneas were swollen, these values represented the best that could be obtained given the current protocol for obtaining human tissue through the eye bank.

Corneas also were obtained from New Zealand white rabbits that were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Each freshly excised cornea, with the epithelium intact, was mounted in the in vitro specular microscope, and the endothelium was perfused with glutathione bicarbonate Ringer under the same conditions as the human corneas except that the perfusion time varied. Specimen HE78 was perfused for 3 hours, whereas specimens HE34 and HE35 were perfused for 1 hour. The corneas maintained their initial thickness during perfusion and were, therefore, unswollen. The final perfused thicknesses were 0.384 mm, 0.361 mm, and 0.365 mm, respectively.

**Preparation of Electron Micrographs**

After perfusion, the corneas were fixed overnight with 2.5% glutaraldehyde in 0.1 M cacodylate, washed in 0.1 M cacodylate and postfixed with 1% osmium te-
troxide in 0.1 M cacodylate; none were fixed under pressure. The specimens were then stained en bloc with 2% aqueous uranyl acetate, after which they were dehydrated in a series of graded ethanol and placed in resin (LX-112; Ladd Research Industries, Burlington, VT). They were polymerized at 60°C for 2 days. Thick sections were cut with a glass knife on a LKB Model 2128 microtome (LKB-Produkter AB; Bromma, Sweden). Thin sections (60 to 70 nm) were cut on the same microtome but with a diamond knife. In each case, care was taken to avoid oblique cuts. The thin sections were placed on uncoated copper grids and were stained with saturated aqueous phosphotungstic acid for 1 hour and with lead citrate for 30 to 60 seconds. Then they were examined using a JEOL (Tokyo, Japan) 100CX II transmission electron microscope.

Multiple EMs were taken of shallow anterior, middle, and deep posterior regions of the human and rabbit stroma. The shallow anterior stroma was identified by taking views just below Bowman's layer for humans and below the epithelium for rabbits. The middle stroma was identified by counting grid squares across the corneal thickness and choosing views from the middle square. The deep posterior stroma was identified by taking views just in front of Descemet's membrane. In addition, for the human stroma, two more regions were considered: deep anterior stroma and shallow posterior stroma. The known dimensions of the specimen grid were used to locate these regions with respect to the shallow anterior and deep posterior.

Using a light microscope, central corneal thickness of the embedded thick sections was measured and compared to the thickness measured at the end of perfusion. In each case, the final embedded thickness was slightly less than the thickness at the end of perfusion. For the deep epithelialized human corneas HE246, HE194, HE201, and HE245, the final embedded thicknesses were 0.534 mm, 0.588 mm, 0.603 mm, and 0.539 mm, respectively. Thus, assuming that the in vivo deep epithelialized thickness for each of the human corneas was 0.47 mm, the tissue subsequently analyzed was between 14% and 28% swelled.29-37 For the rabbit corneas, the final embedded thicknesses (with the epithelium intact) were 0.353 mm, 0.316 mm, and 0.324 mm for corneas HE78, HE34, and HE35, respectively. Thus, the rabbit tissue was slightly (~10%) shrunken from the true thickness.

Image Analysis
Electron micrographs ranging in magnification from ×47,500 to ×53,200 were selected for analysis. Only EMs that had at least one lamella containing fibrils whose cross-sections appeared circular were used. The largest such region for each EM was then digitized on either an Optronics International (Chelmsford, MA) P-1700 or a Howtek (Hudson, NH) D4000 rotating drum scanner. When using the Optronics rotating drum scanner, a pixel size of 200 μm was used initially for three of the EMs, but subsequent EMs were digitized using a pixel size of 100 μm. In the tissue, these pixel sizes corresponded to a square region approximately 4 nm and 2 nm on a side, respectively. Electron micrographs scanned on the Howtek rotating drum scanner were digitized using a pixel size of 101.6 μm (250 dpi). To check that the scanners gave the same results, two EMs were digitized on both and then analyzed. The results were in excellent agreement. The scan data were then passed to a Macintosh Ilfx or Quadra 950 (Apple; Cupertino, CA), on which they were viewed and processed using a modified version of the program NIH.Image 1.28. The details of the methods used to locate the positions of the fibril centers and to determine the fibril radii have been described in detail elsewhere.31

Theory
Light that exits an object in the same direction it entered is said to be undeviated light. The fraction of light transmitted undeviated through the cornea is related to the total scattering cross-section per fibril per unit length, σr, by the equation

\[ F_r = e^{-\Delta \sigma_r} \quad (1) \]

where Δ is the thickness of the stroma, and ρ is the number density of fibrils in the stroma. The total scattering cross-section is a function of light wavelength, λ, and the size, shape and composition of the scatterer.18 It can be written in the form

\[ \sigma_r(\lambda) = \langle \sigma_{0r}(\lambda) \rangle \sigma_{0f}(\lambda) \quad (2) \]

where \( \langle \sigma_{0r}(\lambda) \rangle \) is the total scattering cross-section of an average isolated fibril, and \( \sigma_{0f} \) is the interference factor used to measure the degree of order in the spatial arrangement of fibril axes and of fibril sizes.21 Each of these factors will be discussed in turn.

For weak scatterers, the total scattering cross-section per unit length for an isolated fibril is given by

\[ \sigma_{0f}(\lambda) = \frac{n_r^2 \pi^3 (\pi d_f)^2 (m^2 - 1)^2}{\lambda^3} \left[ 1 + \frac{2}{(m^2 + 1)^2} \right] \quad (3) \]

where \( n_r \) is the refractive index of the ground sub-

* The program NIH.Image was written by Wayne Rasband of the National Institutes of Health. A copy, along with extensive documentation, may be obtained by writing him at National Institutes of Health, Bldg. 36, Room 2A-03, Bethesda, MD 20892 or by contacting him at the following electronic mail addresses: Internet: wayne@helix.nih.gov; or CompuServe: >INTERNET: wayne@helix.nih.gov.
stance, \( m \) is the fibril's refractive index relative to \( n_g \), \( a \) is the radius of the fibril, and the incident light is assumed to be unpolarized.\(^{21}\) For an assembly of fibrils of varying diameters, the average isolated fibril cross-section \( \langle a, n_g(\lambda) \rangle \) is obtained by summing the value of equation 3 for each fibril and dividing the sum by the number of fibrils, \( N \). Estimates for \( n_g \) and \( m \) are obtained using the Gladstone-Dale law of mixtures and the refractive increment law. Both laws, along with numeric values of their constituents, have been described previously.\(^{17,23,28,40}\) The necessary results are

\[
n_g = n_a + \frac{C_0 R_0}{1 - AF}
\]  

(4)

and, under the assumption that each fibril has the same amount of collagen,

\[
n_f = n_a + (n_c - n_a) \frac{A_{f_D}}{\rho A_f}
\]

(5)

where \( n_a (=1.335) \) is the index of refraction of aqueous, \( C_0 (=0.063) \) is the mass concentration of noncollagenous organic material in the stroma, \( R_o (=0.18) \) is the specific refractive increment, \( AF \) is the area fraction of the stroma occupied by fibrils, \( A_{f_D} (=0.1175) \) is the area fraction of dry collagen, \( A_f \) is the cross-sectional area of the specific fibril under consideration, \( n_c (=1.547) \) is the index of refraction of dry collagen, and \( n_f \) is the index of refraction of the hydrated fibril; \( m \) is determined by dividing \( n_f \) by \( n_c \). The value for \( AF \) was determined directly for each EM and is equal to the average cross-sectional area of a fibril, \( \pi(a^2) \), times the fibril number density \( \rho \). The area fraction of dry collagen was derived from the experimental values of the mass fraction of collagen and the.

**FIGURE 1.** Electron micrographs from the anterior (top), middle (center), and posterior (bottom) stroma of a human cornea (HE201). The regions shown correspond closely to those analyzed for the calculations. Micrographs from the other corneas were similar. Scale bar = 1 \( \mu \)m.
specific gravity of collagen and stroma.\textsuperscript{23,28} The area of the J\textsuperscript{th} fibril is $\pi a_J^2$, thus, $n_J$ and therefore $m_J$ varies from fibril to fibril. For independent scatterers, the interference factor in equation 2 is unity, and the total scattering cross-section per fibril per unit length reduces to the average isolated fibril cross-section, $\langle \sigma_0(\lambda) \rangle$.

The first step toward obtaining the interference factor in equation 2 was to note that the total scattering cross-section can be obtained by integrating $\sigma(\lambda, \theta_J)$, the angular (or differential) scattering cross-section, over all scattering angles, which, for long cylinders, leads to

$$
\sigma(\lambda) = \int_0^{2\pi} \sigma(\lambda, \theta_J) d\theta_J.
$$

The angular cross-section is proportional to the absolute square of the field scattered into the small angular acceptance $d\theta_J$ at $\theta_J$. This field is the sum of the fields, including the appropriate phase factors, scattered by the fibrils in the entire illuminated region of the cornea divided by the total number of fibrils. If the positions of the fibrils were uncorrelated, then the absolute square of the sum would reduce to the sum of the differential cross-sections of the individual isolated fibrils. For a fibril of radius $a_J$, the differential scattering cross-section per unit length for an isolated fibril\textsuperscript{21,27} is given by

$$
\sigma_0(\lambda, \theta_J) = \frac{n_J^2 \pi a_J^2 (m_J^2 - 1)^2}{2\lambda^3} \left[ 1 + \frac{4 \cos^2 \theta_J}{(m_J^2 + 1)^2} \right].
$$

If the amount of collagen in each fibril is constant, then differences in fibril radii are caused by differences in hydration. In that case, the relative refractive index, $m_J$, will depend on fibril radius, and the average isolated fibril differential scattering cross-section, $\langle \sigma_0(\lambda, \theta_J) \rangle$, is obtained by summing equation 7 over fibrils and dividing by the number of fibrils.

In general, the fibril positions are correlated, and the differential scattering cross-section per fibril per unit length is written in the form

$$
\sigma(\lambda, \theta_J) = \langle \sigma_0(\lambda, \theta_J) \rangle \mathcal{F}(q),
$$

where $\mathcal{F}(q)$ is a generalized structure factor that takes into account interference between the fields scattered by different fibrils. The structure factor that is usually defined\textsuperscript{41,42} assumes that all scatterers (fibrils) are identical and, hence, that the field scattered by each fibril is the same. By including the effects of variable fibril diameter and index of refraction, the generalized structure factor defined here explicitly accounts for nonidentical fibrils and allows for the general case in which the field scattered by each fibril is different.

For fibrils of uniform diameter, this generalized structure factor reduces to the one usually defined\textsuperscript{41,42}. The vector $q$ is defined as

$$
q = k_i - k_s,
$$

where $k_i$ is the incident wave vector whose direction is in the propagation direction of the incident light, and $k_s$ is the scattered wave vector whose direction is in the direction of the scattered light (i.e., $\theta_J$); both $k_i$ and $k_s$ have a magnitude of $2\pi n_J/\lambda$. The magnitude of $q$ is given by $4\pi n_J \sin (\theta_J/2)/\lambda$.

The task faced by light scattering theories is to estimate this structure factor for the entire illuminated cornea from the limited information contained in the EMs that depict the structures in a tiny region—typically, a few microns on a side. The direct summation of fields method enables one to estimate the ensemble
where the overbars denote sample averages and $N$ is the number of fibrils within a grid element. The quantity $S_b(q)$ is given by

$$S_b(q) = \frac{N(b)}{\sum_{j=1}^{N(b)} E_j(\lambda, \theta_j) d\theta_j / \langle \sigma_b(\lambda, \theta_j) \rangle^{1/2}}, \tag{11}$$

where the summation is over all the $N(b)$ fibrils within the $b^{th}$ grid element, $r_j$ is the location of the center of the $j^{th}$ fibril, and $E_j(\lambda, \theta_j)$ is the field scattered by it. The explicit functional form for $E_j(\lambda, \theta_j)$ is readily available, but it is not needed here other than to note that the absolute square of $E_j(\lambda, \theta_j)$ is just $\sigma_b(\theta_j)$ as given in equation 7. If the fibrils were all the same size, $E_j(\lambda, \theta_j)$ would be independent of $j$, and $S_b(q)$ would be the standard phase sum. It is of interest to note that in the forward direction, where $\theta_j$ is zero, equation 10 predicts that scattering in the forward direction is related to the fluctuation in the number of fibrils, if all the fibril diameters are equal. For the purposes of this article, the fibril positions and diameters are used to evaluate the structure factor at scattering angles in the interval 0 to $\pi$. These results are then inserted into equation 8 and used to evaluate the integral in equation 6 numerically. The interference factor, $\sigma_\psi$, which was introduced in equation 2, is obtained by dividing the result by $\langle \sigma_\psi(\lambda) \rangle$. Specifically, the interference factor is defined by

$$\sigma_\psi(\lambda) = \frac{1}{\langle \sigma_\psi(\lambda) \rangle} \int_0^{2\pi} \langle \sigma_\psi(\lambda, \theta_j) \rangle F(q) d\theta_j. \tag{12}$$

Image processing techniques are used to obtain fibril positions and radii from structures in EMs of anterior, middle, and posterior regions of endothelial perfused normal rabbit corneas and endothelial perfused human eye bank corneas. These are used to compute $\sigma_\psi$, $\sigma_\theta$, $\sigma_r$, and $F_T$.

RESULTS

Figure 1 shows typical EMs from the anterior, middle, and posterior stroma of one of the human corneas. Similar EMs for one of the rabbit corneas are shown in Figure 2. The results of the EM analysis for the root mean square fibril radius, fibril number density, and

<table>
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<th>Cornea</th>
<th>Age (years)</th>
<th>Thickness (mm)*</th>
<th>Region</th>
<th>Total Fibrils</th>
<th>rms radius ($\sigma^2$) 1/2 Å</th>
<th>$\rho \times 10^6$ Fibrils Å$^{-2}$</th>
<th>Area Fraction</th>
<th>( $\sigma_\psi$ ) $\times 10^2$ Å at 500 nm</th>
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<td></td>
<td></td>
<td></td>
<td>Ant</td>
<td>2271</td>
<td>157 ± 12</td>
<td>5.50</td>
<td>0.427</td>
<td>1.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mid</td>
<td>1914</td>
<td>152 ± 11</td>
<td>4.34</td>
<td>0.318</td>
<td>4.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post</td>
<td>1182</td>
<td>138 ± 12</td>
<td>5.65</td>
<td>0.341</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post</td>
<td>2177</td>
<td>136 ± 9</td>
<td>5.54</td>
<td>0.324</td>
<td>2.53</td>
</tr>
</tbody>
</table>

Ant = anterior; Mid = middle; Post = posterior.

* Final embedded thickness of central deep epithelialized cornea.
area fraction occupied by fibrils are compiled in Tables 1 and 2 for each of the four human and three rabbit corneas analyzed.

Results in Tables 1 and 2 show that for each of the human and rabbit corneas, the number density of fibrils, \( \rho \), is greater in the posterior stroma than in the anterior stroma. The average ratio of posterior density to anterior density is 1.39 ± 0.01 for the three rabbit corneas and 1.12 ± 0.06 for the four human corneas. The number density of fibrils in the mid-stroma is lower than in the anterior stroma in each of the human corneas, but it is between the anterior and posterior values in each of the rabbit corneas.

Table 1 shows that to within the widths of their distributions, mean fibril radii are essentially constant across the depth of the human cornea. Table 2 shows that in each of the three rabbit corneas, the mean fibril radius in the anterior is systematically greater than it is in the posterior. There are no systematic variations in the mean values of the mid-stromal fibril radii. Figure 3 shows distributions of the fibril radii from the EMs of the human cornea shown in Figure 1, and Figure 4 shows similar data from the EMs of the rabbit cornea shown in Figure 2. The radii distributions for the other human and rabbit corneas are similar. In addition, Tables 1 and 2 show that there are no systematic variations in the area fraction occupied by fibrils across either the human or the rabbit corneas.

Information on the mean spacing of fibrils can be gleaned from the radial distribution function for fibril centers. Figures 5 and 6 show the radial distribution functions obtained from the EM in Figures 1 and 2. These radial distribution functions are characteristic

<table>
<thead>
<tr>
<th>Cornea</th>
<th>Mass (kg)</th>
<th>Thickness (mm)*</th>
<th>Region</th>
<th>Total Fibrils</th>
<th>rms radius ( (a^2)^{1/2} ) Å</th>
<th>( \rho \times 10^6 ) Fibrils ( \text{Å}^2 )</th>
<th>Area Fraction</th>
<th>( \langle \sigma_w \rangle \times 10^2 ) Å at 500 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE78</td>
<td>3.0 ± 0.5</td>
<td>0.353</td>
<td>Ant</td>
<td>1002</td>
<td>194 ± 13</td>
<td>3.95</td>
<td>0.467</td>
<td>2.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mid</td>
<td>1568</td>
<td>174 ± 14</td>
<td>4.47</td>
<td>0.426</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post</td>
<td>2896</td>
<td>162 ± 12</td>
<td>5.48</td>
<td>0.454</td>
<td>1.64</td>
</tr>
<tr>
<td>HE34</td>
<td>3.0 ± 0.5</td>
<td>0.316</td>
<td>Ant</td>
<td>1737</td>
<td>189 ± 10</td>
<td>4.04</td>
<td>0.453</td>
<td>3.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mid</td>
<td>2695</td>
<td>197 ± 10</td>
<td>3.56</td>
<td>0.433</td>
<td>4.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post</td>
<td>2434</td>
<td>161 ± 10</td>
<td>5.59</td>
<td>0.455</td>
<td>1.57</td>
</tr>
<tr>
<td>HE35</td>
<td>3.0 ± 0.5</td>
<td>0.324</td>
<td>Ant</td>
<td>1518</td>
<td>185 ± 14</td>
<td>3.63</td>
<td>0.585</td>
<td>4.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mid</td>
<td>1218</td>
<td>196 ± 11</td>
<td>4.10</td>
<td>0.496</td>
<td>2.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Post</td>
<td>2642</td>
<td>171 ± 10</td>
<td>5.06</td>
<td>0.465</td>
<td>1.81</td>
</tr>
</tbody>
</table>

* Final embedded thickness of central cornea with epithelium intact.

Ant = anterior; Mid = middle; Post = posterior.
of fibrils that have short-range order.\textsuperscript{16,17,26} Tables 3 and 4 list the nearest neighbor distances (defined as the value of \( r \) at the first maximum of \( g(r) \)) for each of the human and rabbit corneas, respectively. There are no systematic variations in nearest neighbor distances from anterior to posterior in the human corneas.

The values of the average isolated (single) fibril total scattering cross-section, \( \langle \sigma_o(\lambda) \rangle \), at the wavelength 500 nm are listed in Tables 1 and 2 for humans and rabbits, respectively. Values at other wavelengths are not given because, as seen from equation 3, only the factor \( 1/\lambda^3 \) contributes to wavelength dependence. Thus, a value for \( \langle \sigma_o(\lambda) \rangle \) at any desired light wavelength \( \lambda \) (in nanometers) can be obtained by multiplying the values given in the tables by the factor \((500/\lambda)^3\). The average value of \( \langle \sigma_o(\lambda) \rangle \) for the anterior and posterior regions is obtained by averaging results from the EMs analyzed. For humans, the results at 500 nm are \((3.15 \pm 1.14) \times 10^{-2}\text{Å} \) for the anterior and \((2.35 \pm 0.27) \times 10^{-2}\text{Å} \) for the posterior. A paired \( t \)-test\textsuperscript{43} indicates that this difference is not significant.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Distribution of fibril radii in the anterior (a), middle (b), and posterior (c) stroma of the rabbit cornea pictured in Figure 2. These results were obtained using the image analysis methods described in the Methods section.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Radial distribution functions for the fibrils in the anterior (a), middle (b), and posterior (c) stroma of the human cornea pictured in Figure 1. The function \( g(r) \) represents the relative probability of finding fibril centers at a distance \( r \) from any reference fibril.}
\end{figure}
Ultrastructure in Anterior and Posterior Stroma

2.5
2
1.5
1
0.5
0
0 500 1000 1500 2000 2500
r (Å)

FIGURE 6. Radial distribution functions for the fibrils in the anterior (a), middle (b), and posterior (c) stroma of the rabbit cornea pictured in Figure 2. The function \( g(r) \) represents the relative probability of finding fibril centers at a distance \( r \) from any reference fibril.

\( P > 0.07 \). The corresponding results for rabbit are \((3.63 \pm 1.12) \times 10^{-2} \text{Å} \) for the anterior and \((1.67 \pm 0.12) \times 10^{-2} \text{Å} \) for the posterior; because of the small sample size, no statistical tests were performed.

The results for the interference factor, \( \sigma_{\infty} \), the total scattering cross-section, \( \sigma_t \), and the fraction of light transmitted, \( F_T \), which were discussed under Theory in the Methods section, are presented in Figures 7 through 13. The error bars in each of these figures represent the standard error of the mean. We will present each result separately. However, we note here that no statistical testing was performed on the rabbit data because of the small number (three) of EMs analyzed from each region.

Figure 7A is a plot of the interference factor, \( \sigma_{\infty}(\lambda) \) (see equation 12), for the anterior and posterior regions of each of the human corneas. There is a clear separation of values between the anterior and posterior regions of the corneas. For each of corneas HE246 (squares) and HE201 (circles), both EMs from the posterior clearly show more order than the two corresponding EMs from the anterior. In each of corneas HE245 (triangles) and HE194 (diamonds), the two EMs from the posterior clearly show more order than one of the EMs from the anterior, and, for cornea HE245, both posterior EMs are marginally

TABLE 3. Human Stroma Refractive Indices*  

<table>
<thead>
<tr>
<th>Cornea</th>
<th>Region</th>
<th>( n_f )</th>
<th>( m )</th>
<th>Nearest Neighbor Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE246</td>
<td>Ant</td>
<td>1.353</td>
<td>1.035</td>
<td>399</td>
</tr>
<tr>
<td></td>
<td>Mid</td>
<td>1.354</td>
<td>1.032</td>
<td>405</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>1.352</td>
<td>1.040</td>
<td>434</td>
</tr>
<tr>
<td>HE194</td>
<td>Ant</td>
<td>1.351</td>
<td>1.047</td>
<td>442</td>
</tr>
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<td></td>
<td>Mid</td>
<td>1.351</td>
<td>1.048</td>
<td>446</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>1.353</td>
<td>1.096</td>
<td>385</td>
</tr>
<tr>
<td>HE201</td>
<td>Ant</td>
<td>1.352</td>
<td>1.043</td>
<td>412</td>
</tr>
<tr>
<td></td>
<td>Mid</td>
<td>1.351</td>
<td>1.051</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>1.353</td>
<td>1.036</td>
<td>425</td>
</tr>
<tr>
<td>HE245</td>
<td>Ant</td>
<td>1.353</td>
<td>1.058</td>
<td>412</td>
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<tr>
<td></td>
<td>Mid</td>
<td>1.352</td>
<td>1.046</td>
<td>449</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>1.355</td>
<td>1.028</td>
<td>405</td>
</tr>
</tbody>
</table>

Ant = anterior; Mid = middle; Post = posterior.

* The fibril refractive index may be obtained from the relation \( n_f = n_m \).

TABLE 4. Rabbit Stroma Refractive Indices*  

<table>
<thead>
<tr>
<th>Cornea</th>
<th>Region</th>
<th>( n_f )</th>
<th>( m )</th>
<th>Nearest Neighbor Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE78</td>
<td>Ant</td>
<td>1.356</td>
<td>1.024</td>
<td>466</td>
</tr>
<tr>
<td>Mid</td>
<td>1.355</td>
<td>1.028</td>
<td>476</td>
<td></td>
</tr>
<tr>
<td>Post</td>
<td>1.356</td>
<td>1.025</td>
<td>433</td>
<td></td>
</tr>
<tr>
<td>HE34</td>
<td>Ant</td>
<td>1.356</td>
<td>1.025</td>
<td>472</td>
</tr>
<tr>
<td>Mid</td>
<td>1.355</td>
<td>1.027</td>
<td>529</td>
<td></td>
</tr>
<tr>
<td>Post</td>
<td>1.356</td>
<td>1.025</td>
<td>414</td>
<td></td>
</tr>
<tr>
<td>HE35</td>
<td>Ant</td>
<td>1.353</td>
<td>1.035</td>
<td>529</td>
</tr>
<tr>
<td>Mid</td>
<td>1.358</td>
<td>1.020</td>
<td>472</td>
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</tr>
<tr>
<td>Post</td>
<td>1.356</td>
<td>1.024</td>
<td>433</td>
<td></td>
</tr>
</tbody>
</table>

Ant = anterior; Mid = middle; Post = posterior.

* The fibril refractive index may be obtained from the relation \( n_f = n_m \).
more ordered than the other EM from the anterior, but the difference is not as distinct. Only in cornea HE194 was one of the EMs from the anterior (marginally) more ordered than both posterior EMs. Values for the mid-stroma (not shown) spanned the range between the posterior and anterior values. Figure 7B shows the average values of $\sigma_w(\lambda)$ for the anterior and posterior regions. Clearly, the average value for $\sigma_w(\lambda)$ is lower for the posterior region than it is for the anterior, indicating that the posterior cornea was more ordered than the anterior. A paired $t$-test showed that this difference is significant ($P < 0.004$) for all wavelengths. A Wilcoxon signed rank test also indicated this difference to be significant ($P < 0.012$) at all wavelengths. Figures 8A and 8B show the corresponding results for the rabbit corneas. The posterior region of each rabbit cornea is more highly ordered than the anterior, and the values for the mid-stroma (not shown) span the range between those of the anterior and the posterior. The fibrils in rabbit cornea are more ordered than those in corresponding regions of human cornea; anterior rabbit cornea has approximately the same degree of ordering as posterior human cornea.

Figures 9 and 10 are plots of $\sigma(\lambda)$ for the human and rabbit corneas, respectively. In humans, the ratio of the anterior total scattering cross-section to that of the posterior was approximately 2, ranging from approximately 2.1 at 400 nm to 2.4 at 750 nm. Both a paired $t$-test ($P < 0.02$) and a Wilcoxon signed rank test ($P < 0.012$) indicate that this difference is significant at each of the wavelengths. In rabbit, the anterior total scattering cross-section is approximately three times greater than that of the posterior at all wavelengths. Because the interference factor is nearly independent of wavelength in the visible portion of the spectrum, virtually all the total scattering cross-sec-
Ultrastructure in Anterior and Posterior Stroma

Figure 9. Average value of the total fibril scattering cross-section per unit length, \( \sigma_r(\lambda) \), for anterior and posterior regions of the human corneas.

The transparency of a cornea would have if it had the structure and properties of the anterior or posterior regions can be computed using equation 1. The results for the average value of \( F_T \) are shown in Figures 11 and 12 for human and rabbit corneas, respectively. Even though the fibril number density is roughly 10% greater in the posterior of human cornea and 40% greater in the posterior of rabbit cornea, the factors of 2 and 3 in the differences in scattering between the two regions cause the anterior to be less transparent in both species. For humans, a paired \( t \)-test indicated that the difference in transparency between the anterior and posterior was significant at each wavelength \( (P < 0.015) \); a Wilcoxon signed rank test also indicated significance \( (P < 0.012) \) for each wavelength.

At this point, recall that the calculations for each EM were performed on the assumption that each fibril contained a fixed amount of collagen. Instead, one could make a different assumption—that each fibril has a fixed percent of collagen. In this case, fibril hydration would be independent of fibril radius, and it can be readily shown that each fibril would have the same index of refraction. Tables 3 and 4 list the values of the relative index of refraction for the fibrils for each EM computed under this assumption. Calculations using the distribution of fibril radii (assuming each fibril contains a fixed percent of collagen) also yield results that show the posterior to be more ordered and more transparent than the anterior, for humans and rabbits. However, as seen in Figure 13, the difference in \( \sigma_r(\lambda) \) for human cornea is much less distinct and, in fact, is not significant as determined by either a paired \( t \)-test or a Wilcoxon signed rank test. Similar small differences in \( \sigma_r(\lambda) \) and \( F_T \) were not statistically significant. In comparing Figure 13a with Figure 7a, one sees that for each anterior EM, the interference factor (under the assumption of a fixed percent

Figure 10. Average value of the total fibril scattering cross-section per unit length, \( \sigma_r(\lambda) \), for anterior and posterior regions of the rabbit corneas.

Figure 11. Average human cornea transmittance computed with the assumption that the entire cornea has the structure and properties of its anterior or posterior region. The quantity plotted is \( F_T = \exp(-\langle \sigma_r(\lambda) \rangle A) \) (see equation 1), where the thickness \( \Delta = 0.55 \text{ mm} \).

Figure 12. Average rabbit cornea transmittance computed assuming the entire cornea has the structure and properties of its anterior or posterior region. The quantity plotted is \( F_T = \exp(-\langle \sigma_r(\lambda) \rangle A) \) (see equation 1), where the thickness \( \Delta = 0.38 \text{ mm} \).
more ordered and more transparent. A paired t-test showed the results to be significant in this case for the difference in $\sigma_{in}$ ($P < 0.02$), $\sigma_\lambda$ ($P < 0.05$), and $F_T$ ($P < 0.05$). Again, a Wilcoxon signed rank test also indicates significance for $\sigma_{in}$ ($P < 0.025$), $\sigma_\lambda$ ($P < 0.035$), and $F_T$ ($P < 0.025$).

Finally, we note that some of the high magnification EMs from corneas HE245 and HE24 showed features that appeared similar to lipid deposits. These corneas were from the same donor and were not eligible for transplant because the patient had sepsis at death. Inspection of nine low magnification ($\times 9000$) EMs from each cornea showed deposits in three of the EMs from cornea HE245 and in one of the EMs from cornea HE246. Further analysis showed that the area fraction of the deposits ranged between roughly 0.3% and 0.7% of the total area of the EMs. Deposits

![Figure 13](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933190/)

**FIGURE 13.** (A) The normalized total scattering cross-section, $\sigma_{in}(\lambda)$, calculated using the assumption that each fibril had a fixed percent of collagen. Two electron micrographs from anterior stroma and two from posterior stroma were analyzed for each cornea. Open symbols = electron micrographs from the anterior stroma; solid symbols = electron micrographs from the posterior stroma. These symbols correspond with those in Figure 7A. Smaller values of $\sigma_{in}(\lambda)$ correspond to more ordered fibril arrangements. (B) Average value of $\sigma_{in}(\lambda)$ for the anterior and posterior regions of the four human corneas.

collagen) decreased in every instance from the value found, assuming a fixed amount of collagen. The result is seen for six of the posterior EMs, but in the other two posterior EMs, there was a significant increase in $\sigma_{in}$. There was virtually no change in the average isolated fibril scattering cross-section, $\langle \sigma_{0} \rangle$, between the two assumptions in any of the EMs.

The simplest approximation one could make would be to ignore the distribution of fibril radii altogether and assume all fibril radii are equal. If, for each EM, one chooses the radius associated with the average fibril area (i.e., $\langle \dot{d} \rangle^{1/2}$), the results for human and rabbit corneas are similar to the results computed using the distribution of fibril radii and our original assumption that each fibril contains a fixed amount of collagen, namely, that there is a distinction between the anterior and posterior regions, with the posterior

![Figure 14](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933190/)

**FIGURE 14.** Electron micrograph from posterior of cornea HE245 showing deposits. The large rectangle shows the region analyzed, which includes deposits. The smaller rectangle shows the normal-looking region that was analyzed. Scale bar = 1 μm.
We have used information obtained from image analysis of EMs of the anterior, middle, and posterior stroma to investigate possible differences in the fibrillar ultrastructure and their influence on predicted light scattering and transparency. The direct summation of fields method was used to calculate the total scattering cross-section per fibril per unit length, \( \sigma,(\lambda) \). We separated \( \sigma,(\lambda) \) into a product of two factors, \( \sigma,0(\lambda) \), the average scattering cross-section for an isolated fibril, which does not depend on the fibril position, and \( \sigma,0(\lambda) \), which measures the reduction of the scattering cross-section from destructive interference between the scattered fields and depends on the degree to which the fibrils are ordered. This separation facilitated comparisons of the different stromal regions.

In human stroma, the interference factor, \( \sigma,0(\lambda) \), is approximately 0.12 in the posterior and approximately 0.19 (ranging from 0.16 at 400 nm to 0.22 at 750 nm) in the anterior and is essentially independent of wavelength. In rabbits, it is approximately 0.085 in the posterior stroma and approximately 0.11 in the anterior stroma. Because the extent to which the interference factor deviates from unity is a measure of the spatial distribution of fibrils and, to a lesser extent, the spatial distribution of fibril scattering strengths, its small magnitude indicates that there is substantial fibrillar ordering throughout the stromas of both species; however, fibrils in posterior stroma were found to be more ordered than those in anterior stroma. These results indicate that there is a difference between ultrastructure in anterior and posterior stroma from endothelial perfused normal rabbit corneas and endothelial perfused human eye bank corneas.

However, in view of the fact that the human corneas were swollen at the end of perfusion (see Methods), the question arose as to whether these results were attributable to some factor related to swelling differences between anterior and posterior regions. To address this question, we first note that the salient feature of swollen corneas is the presence of regions devoid of fibrils,20,22 often referred to as “lakes.” The effect of lakes is to increase scattering and to alter the wavelength dependence of the total scattering cross-section by adding a term \( 1/\lambda^2 \) that is proportional to 1/\( \lambda^2 \). Because the isolated fibril total scattering cross-section is proportional to 1/\( \lambda^3 \) (see equation 3), the effect of lakes on the normalized total scattering cross-section is to make it proportional to the incident light wavelength \( \lambda \). The direct summation of fields method is sensitive enough to detect this effect.44 Inspection of Figure 7A shows that six of the eight anterior plots have small positive slopes, whereas none of the posterior plots have significant positive slopes. Figure 7B reflects the same results for the average value of \( \sigma,0(\lambda) \). These results indicate that the anterior region of the human corneas may have been slightly swollen. Thus, it is possible that differences in swelling between anterior and posterior stroma could explain the ultrastructural differences we noted between the anterior and posterior regions of human corneas. On the other hand, the results found for rabbits indicated a difference between anterior and posterior stromal ultrastructure similar to that seen for humans. However, as discussed in the Methods section, the thickness of the rabbit corneas was unaltered during perfusion and was slightly (\( \approx 10\% \)) shrunken after fixation. Furthermore, inspection of Figures 8A and 8B shows no appreciable slope for any of the rabbit results. Consequently, it is unlikely that the ultrastructural differences seen between anterior and posterior rabbit stroma can be attributed to differences in swelling between the two regions.

We also found that the ordering throughout the stroma is short-ranged and extended only to two or three of the nearest neighbors.16–19 No systematic variations in nearest neighbor distances from anterior to posterior were found in human corneas. This finding is in agreement with Patey et al,32 who found that the mean interfibrillar spacing measured from EM did not vary from anterior to posterior stroma in human cornea. Their values for the mean interfibrillar spacing are also in close agreement with the nearest neighbor distances in Table 3; however, the distances in Table 3 are approximately 20% smaller than those deduced from synchrotron x-ray diffraction45 in humans and were slightly smaller than the mean interfibrillar spacing in humans measured from EM by Giraud et al.39 In all rabbit corneas, the nearest neighbor distance was consistently greater in the anterior than in the posterior stroma, as might be expected from the anterior–posterior variation in fibril number density. There was no systematic variation of nearest neighbor distance in the midstromal region. The nearest neighbor distances seen in Table 4 are smaller than the mean interfibrillar spacing found by Giraud et al for

**DISCUSSION**

We have used information obtained from image analysis of EMs of the anterior, middle, and posterior stroma to investigate possible differences in the fibrillar ultrastructure and their influence on predicted light scattering and transparency. The direct summation of fields method was used to calculate the total scattering cross-section per fibril per unit length, \( \sigma,(\lambda) \). We separated \( \sigma,(\lambda) \) into a product of two factors, \( \sigma,0(\lambda) \), the average scattering cross-section for an isolated fibril, which does not depend on the fibril position, and \( \sigma,0(\lambda) \), which measures the reduction of the scattering cross-section from destructive interference between the scattered fields and depends on the degree to which the fibrils are ordered. This separation facilitated comparisons of the different stromal regions.

In human stroma, the interference factor, \( \sigma,0(\lambda) \), is approximately 0.12 in the posterior and approximately 0.19 (ranging from 0.16 at 400 nm to 0.22 at 750 nm) in the anterior and is essentially independent of wavelength. In rabbits, it is approximately 0.085 in the posterior stroma and approximately 0.11 in the anterior stroma. Because the extent to which the interference factor deviates from unity is a measure of the spatial distribution of fibrils and, to a lesser extent, the spatial distribution of fibril scattering strengths, its small magnitude indicates that there is substantial fibrillar ordering throughout the stromas of both species; however, fibrils in posterior stroma were found to be more ordered than those in anterior stroma. These results indicate that there is a difference between ultrastructure in anterior and posterior stroma from endothelial perfused normal rabbit corneas and endothelial perfused human eye bank corneas.

However, in view of the fact that the human corneas were swollen at the end of perfusion (see Methods), the question arose as to whether these results were attributable to some factor related to swelling differences between anterior and posterior regions. To address this question, we first note that the salient feature of swollen corneas is the presence of regions devoid of fibrils,20,22 often referred to as “lakes.” The effect of lakes is to increase scattering and to alter the wavelength dependence of the total scattering cross-section by adding a term \( 1/\lambda^2 \) that is proportional to 1/\( \lambda^2 \). Because the isolated fibril total scattering cross-section is proportional to 1/\( \lambda^3 \) (see equation 3), the effect of lakes on the normalized total scattering cross-section is to make it proportional to the incident light wavelength \( \lambda \). The direct summation of fields method is sensitive enough to detect this effect.44 Inspection of Figure 7A shows that six of the eight anterior plots have small positive slopes, whereas none of the posterior plots have significant positive slopes. Figure 7B reflects the same results for the average value of \( \sigma,0(\lambda) \). These results indicate that the anterior region of the human corneas may have been slightly swollen. Thus, it is possible that differences in swelling between anterior and posterior stroma could explain the ultrastructural differences we noted between the anterior and posterior regions of human corneas. On the other hand, the results found for rabbits indicated a difference between anterior and posterior stromal ultrastructure similar to that seen for humans. However, as discussed in the Methods section, the thickness of the rabbit corneas was unaltered during perfusion and was slightly (\( \approx 10\% \)) shrunken after fixation. Furthermore, inspection of Figures 8A and 8B shows no appreciable slope for any of the rabbit results. Consequently, it is unlikely that the ultrastructural differences seen between anterior and posterior rabbit stroma can be attributed to differences in swelling between the two regions.

We also found that the ordering throughout the stroma is short-ranged and extended only to two or three of the nearest neighbors.16–19 No systematic variations in nearest neighbor distances from anterior to posterior were found in human corneas. This finding is in agreement with Patey et al,32 who found that the mean interfibrillar spacing measured from EM did not vary from anterior to posterior stroma in human cornea. Their values for the mean interfibrillar spacing are also in close agreement with the nearest neighbor distances in Table 3; however, the distances in Table 3 are approximately 20% smaller than those deduced from synchrotron x-ray diffraction45 in humans and were slightly smaller than the mean interfibrillar spacing in humans measured from EM by Giraud et al.39 In all rabbit corneas, the nearest neighbor distance was consistently greater in the anterior than in the posterior stroma, as might be expected from the anterior–posterior variation in fibril number density. There was no systematic variation of nearest neighbor distance in the midstromal region. The nearest neighbor distances seen in Table 4 are smaller than the mean interfibrillar spacing found by Giraud et al for
rabbitts and are smaller than the fibrillar spacing deduced from synchrotron x-ray diffraction.

Differences in the degree of spatial ordering are particularly interesting when they are examined in the context of other known differences in anterior–posterior properties, particularly those associated with proteoglycans. Proteoglycans, consisting of a peptide core and one or more covalently bound glycosaminoglycan side chains, are a significant component of the inter-fibrillar matrix in the corneal stroma. The two major types of proteoglycans in corneal stroma, named after their glycosaminoglycan side chains, are keratan sulfate proteoglycan and dermatan sulfate proteoglycan. Because of their specific interactions with collagen fibrils, these proteoglycans are thought to play a role in determining the nature of the ordering in fibrillar positions about one another. Casnoto et al showed that in bovine cornea, the ratio of keratan sulfate proteoglycan to dermatan sulfate proteoglycan is greater in the posterior stroma. Moreover, the preferential loss of keratan sulfate from edematous rabbit corneas is said to reflect its having a higher concentration in the posterior stroma. Because keratan sulfate proteoglycan has greater ability to absorb water than dermatan sulfate proteoglycan, data from bovine and rabbit corneas showing that the posterior stroma is more hydrated than the anterior also is consistent with a greater posterior concentration of keratan sulfate proteoglycan. Keratan sulfate proteoglycan is thought to be important in maintaining transparency. Indeed, cloudiness in corneas with macular corneal dystrophy has been associated with their failure to synthesize mature keratan sulfate proteoglycans. Thus, the higher degree of spatial ordering of posterior fibrils may be associated with the reported higher concentration of keratan sulfate proteoglycan in that region.

In human corneas, we found that the radii of the posterior fibrils were approximately 4% smaller on average than those in the anterior, whereas in rabbits they were approximately 15% smaller. Similar differences have been noted before. The values found here for the human fibril radii are approximately 30% greater than some reported values, but they are in close agreement with diameters deduced from synchrotron x-ray diffraction. The differences possibly are due to differences in the preparation method for transmission electron microscopy. A wide range of fibril radii have been reported for rabbit cornea; these found here agree with the larger reported values. Again, these differences have been ascribed to different preparation methods. Recent studies indicate that fibril size may be regulated by small differences in the amount of type V collagen incorporated in the fibrils, which consist predominately of type I collagen. Thus, it is possible that the ratio of type V to type I collagen varies across the stroma; however, based on the data reported here, any measurable variation would most likely be more easily found in rabbit stroma.

In conclusion, we note that our analysis of EM from perfused normal rabbit corneas and perfused human eye bank corneas has shown that all three regions of the stroma are highly transparent and that destructive interference effects due to the short-range spatial ordering among the fibrils play a pivotal role. These effects reduce scattering and increase transparency. Our comparison of the anterior, middle, and posterior stroma shows that the destructive interference effects are greatest in the posterior and least in the anterior stroma for rabbits and humans. However, for human corneas, we emphasize that it is unclear whether these findings are caused by intrinsic ultrastructural differences between the anterior and posterior stroma present in vivo or whether they can be explained by a difference in swelling between the two regions in vitro. We further note that although the final embedded thickness of the human corneas we analyzed was swollen relative to the accepted value for normal human corneas, the final embedded thickness of the rabbit corneas was shrunken (thinner) relative to the actual thickness. Thus, one must be careful about drawing quantitative conclusions about comparisons between humans and rabbits. For example, even though the ultrastructural differences seen between anterior and posterior stroma are qualitatively the same for humans and rabbits, the fact that we found fibrils in rabbit stroma to be more ordered than those in human stroma may be attributable to differences in the state of the final embedded tissue rather than to a true quantitative difference between species.

Key Words

corneal stroma, fibril statistics, light scattering, stromal ultrastructure, transparency

References

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