

3D Collagen Orientation Study of the Human Cornea Using X-ray Diffraction and Femtosecond Laser Technology

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PURPOSE. To study the distribution and predominant orientations of fibrillar collagen at different depths throughout the entire thickness of the human cornea. This information will form the basis of a full three-dimensional reconstruction of the preferred orientations of corneal lamellae.

METHODS. Femtosecond laser technology was used to delaminate the central zones of five human corneas into three separate layers (anterior, mid, and posterior stroma), each with predetermined thicknesses. Wide-angle x-ray diffraction was used to study the gross collagen fibril orientation and distribution within each layer.

RESULTS. The middle and posterior parts of the human cornea demonstrated a preferential orthogonal arrangement of collagen fibrils, directed along the superior-inferior and nasal-temporal meridians, with an increase in the number of lamellae toward the periphery. However, the anterior cornea (33% of total corneal thickness) showed no systematic preferred lamellar orientation.

CONCLUSIONS. In the posterior two thirds of the human cornea, collagen lies predominantly in the vertical and horizontal meridians (directed toward the four major rectus muscles), whereas collagen in the anterior third of the cornea is more isotropic. The predominantly orthogonal arrangement of collagen in the mid and posterior stroma may help to distribute strain in the cornea by allowing it to withstand the pull of the extraocular muscles, whereas the more isotropic arrangement in the anterior cornea may play an important role in the biomechanics of the cornea by resisting intraocular pressure while at the same time maintaining corneal curvature. (*Invest Ophthalmol Vis Sci.* 2009;50:5159-5164) DOI:10.1167/iovs.09-3669

Corneal transparency depends on the unique architecture of the stroma, which accounts for more than 90% of corneal thickness. In humans, the central stroma is made of approximately 200 to 250 stacked lamellae that lie essentially parallel to the surface of the cornea.¹ Adjacent lamellae do not generally lie in the same direction, and the change in orientation between lamellae may be any angle between 0° and 180°. ²⁻⁴ Within a single lamella, collagen fibrils of a uniform diameter lie parallel to one another. They are embedded in a hydrated matrix rich in proteoglycans and salts.^{5,6} The specific arrangement of collagen in the corneal stroma produces destructive interference of light scattered in all directions other than the forward direction, leading to tissue transparency. It is also responsible for maintaining the strength and shape of the cornea.^{7,8}

Many studies have been undertaken to determine the orientation of collagen fibrils within the human cornea.⁸⁻¹³ These studies indicate that, in the human cornea, there is a preferred fibril orientation in the inferior-superior and nasal-temporal directions. This preferred orientation occurs at the center of the cornea and is maintained to within 2 mm of the limbus, where a gradual change to a tangential disposition occurs.⁸ However, the collagen fibril orientation and distribution at different depths within the human cornea has yet to be investigated. In the present study, femtosecond laser technology was used to delaminate human corneas into samples at different depths within the stroma and wide-angle x-ray diffraction was used to study the collagen fibril orientation and distribution within each layer. This is the first step toward a full three-dimensional reconstruction of corneal lamellae preferred orientations. The results of this study should help elucidate the biomechanical origins of some problems induced by excimer laser refractive surgery.

MATERIALS AND METHODS

Samples

All tissue used in this study was obtained in accordance with the tenets of the Declaration of Helsinki. Five human corneas with intact scleral rim were sampled. Three of them (B1-3) were obtained from the Bristol Eye Bank (Bristol, UK) and had previously been stored in culture medium (Eagle's MEM buffered with HEPES and containing 26 mM sodium bicarbonate, 2% fetal bovine serum, 2 mM L-glutamine, penicillin, streptomycin, and amphotericin B) at 37°C. The remaining two corneas (P1 and P2), which were obtained from a pair of donor globes provided by the National Disease Research Interchange (Philadelphia, PA), were transported to Cardiff University on dry ice. Before experimental use, the globes were allowed to thaw at room temperature and the corneas with a scleral rim were removed. Because of variations in postmortem times and storage, some corneas were swollen more than others. An ultrasonic pachymeter showed the measured corneal thickness of the five corneas to range from 570 to 1040 μm (Pachette2; DGH Technology, Exton, PA). However, previous studies have shown that even severe swelling does not change the directions of corneal

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TABLE 1. Stromal Depth Location of the Delaminated Anterior, Middle, and Posterior Layers of Five Studied Corneas

Layer	Cornea				
	B1	B2	B3	P1	P2
Anterior	0–120 (12)	0–140 (14)	0–160 (28)	0–250 (36)	0–200 (33)
Middle	120–240 (12)	140–280 (14)	160–320 (28)	250–500 (36)	200–400 (33)
Posterior	240–1040 (76)	280–990 (72)	320–570 (44)	500–700 (28)	400–600 (33)
Total corneal thickness	1040	990	570	700	600

Data are the distance from the surface of the cornea (μm). Numbers in parentheses represent the percentage of the total corneal thickness included within each layer. Note that corneas B3, P1, and P2 were only slightly swollen compared with B1 and B2, which were swollen to almost twice physiological thickness.

lamellae within the plane of the cornea (Meek KM, et al. *IOVS*. 2005; 46:ARVO E-Abstract 2183). On receipt, all corneas were placed in 4% paraformaldehyde and stored at 4°C until required experimentally.

Each cornea was placed in a sterile Barron chamber (Katena Products Inc., Denville, NJ), and a physiologic saline solution was pumped into the posterior compartment at intraocular pressure to ensure the maintenance of a normal corneal shape and curvature during the delamination process. The chamber and cornea were then positioned under the exit point of a 60-kHz femtosecond laser (FS 60; IntraLase Corp., Irvine, CA), and the appplanation cone of the laser was brought into contact with the corneal surface, causing it to flatten. The laser parameters were set to a flap diameter of 8.5 mm for the anterior stromal layer and 7.5 mm for the mid-stromal layer, a planned flap thickness of between 120 and 250 μm , a side cut angle of 70° , a beam separation of $4 \times 4 \mu\text{m}$ and a cut energy of $4 \mu\text{J}$.

As shown in Table 1, the first delamination, with a diameter of 8.5 mm, was performed between 120 and 250 μm from the corneal surface, thus isolating a sample disc of the anterior stroma. Subsequently, a further delamination, of diameter 7.5 mm, was induced at a depth of 240 to 500 μm from the surface, allowing a second disc to be isolated from the mid stroma. Finally, the residual tissue, measuring between 200 and 800 μm in thickness was isolated with a 12-mm trephine, providing a sample of the posterior stroma complete with a full-thickness scleral rim. Before being placed in fresh 4% paraformaldehyde, each piece of corneal tissue was marked at the 12 o'clock position by means of a small incision.

X-ray Diffraction

To minimize tissue dehydration during x-ray exposure, each piece of corneal tissue was wrapped in thin plastic film (Clingfilm; Superdrug

Stores Plc., Croydon, UK) and placed in an airtight chamber (Perspex; Databank UK, London, UK) with polyester film (Mylar; DuPont-Teijin Films, Middlesbrough, UK) windows. This was then sealed with grease. All x-ray diffraction experiments were performed on station 14.1 at the UK Synchrotron Radiation Source (Daresbury, UK), using a wide-angle beam with a wavelength of 0.1488 nm and a 0.2-mm^2 cross section of the specimen. The x-ray exposure time differed between specimens (25, 35, or 45 seconds) due to variations in the thickness of the corneal tissue and the intensity of the x-ray beam at the time of data collection. X-ray scatter patterns were collected at 0.5-mm intervals over each corneal layer and recorded on a charge-coupled device (CCD) detector (Quantum 4R; ADSC, Poway, CA) located 150 mm behind the specimen. A lead beam stop was positioned between the sample and the detector to stop any undeviated x-rays.

Each x-ray scatter intensity profile was normalized against variations in x-ray exposure time and beam intensity. Figure 1 shows the major steps of x-ray diffraction analysis. A high-angle diffraction pattern from the central human cornea (Fig. 1A) shows the beam-stop position in the center, surrounded by a ring of x-ray scatter corresponding to equatorial scattering from the collagen molecules. Four lobes of increased x-ray scatter intensity can also be seen that correspond to a preponderance of collagen molecules lying in the superior-inferior and nasal-temporal directions. Although the collagen molecules are inclined slightly with respect to the fibril axis, the tilt is less than 15° ,¹⁴ and therefore the molecular alignment can be used as a good indication of the fibril alignment.

In the first stage of the analysis, a radial background scatter (arising from noncollagenous components in the cornea) was subtracted from the reflection by taking 256 segments and fitting a power law function radially outward from the center of the x-ray scatter pattern.^{4,15} After

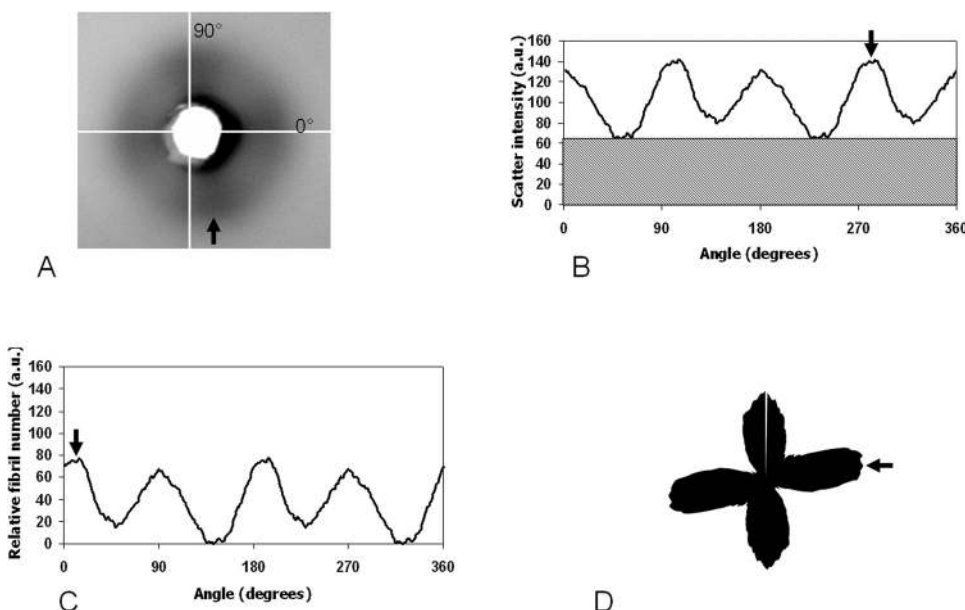


FIGURE 1. Main steps of x-ray diffraction analysis. (A) A high-angle x-ray scatter pattern recorded from the central human cornea. (B) Distribution of scatter intensity around the collagen intermolecular reflection. The total scatter can be divided into scatter from preferentially aligned collagen (clear area) and scatter from isotropically oriented collagen (shaded area). (C) Distribution of scatter intensity after the subtraction of isotropic scatter and a 90° phase shift. Introducing the phase shift effectively converts (B) into a plot of relative fibril number versus angle. (D) The plot in (C) with polar coordinates.

this, the scatter was summed for each radial position, and the summed x-ray scatter intensity plotted as a function of angle (Fig. 1B); the total area under the scatter intensity graph is proportional to the total amount of collagen at that point in the tissue and may be subdivided into x-ray scatter arising from isotropically arranged collagen and scatter from preferentially aligned collagen.

Finally, the isotropic scatter was subtracted from the total scatter intensity profile,^{12,16} and the data shifted by 90° to account for the fact that the x-ray reflections are at right angles to the fibril axes (Fig. 1C). The remaining scatter intensity profile of preferentially aligned collagen was then plotted as polar coordinates (Fig. 1D).¹⁷ Displaying Figure 1C as a polar plot (Fig. 1D) enables the preferred fibril directions that gave rise to the x-ray reflection in Figure 1A to be clearly visualized. The radial size of the plot in a given direction is proportional to the number of fibrils disposed in that direction.

One point in the x-ray pattern is indicated in Figure 1A (arrow) and the arrows in Figure 1B–D allow this point to be followed through each stage of the analysis.

Using this method, each x-ray pattern was analyzed to produce a polar plot representing the amount of collagen preferentially aligned in a given direction or directions, summed throughout the full thickness of the tissue. By recording x-ray patterns across the whole tissue, an assembly of polar plots was then created to visualize the spatial distribution of preferentially aligned collagen fibrils.

To show the relative mass distribution of total and preferentially aligned collagen throughout the tissue, we devised contour maps of x-ray scatter intensity by individually integrating the area under the total x-ray scatter intensity graph (Fig. 1B) and the area under the preferentially aligned collagen x-ray scatter graph (Fig. 1C), to produce single intensity values for each component at every sampling location. These scatter intensity values were then assimilated onto a grid relating to sampling position.

RESULTS

The orientation of collagen fibrils in the anterior one third of cornea P2 is shown in Figure 2A. No consistent preferred orientation was detected in this anterior region (~33% of total corneal thickness). The other four corneas showed similar results in their anterior layers, and, in the case of B1 and B2, a lack of consistent collagen orientation was also seen in the mid-stromal layer of each cornea. However, the results obtained from the mid and posterior stromal layers of corneas B1 and B2 should be treated with caution due to the swollen nature of the tissue. Although, as previously stated, stromal swelling does not affect the predominant orientation of collagen (within the plane of the tissue) (Meek KM, et al. *IOVS* 2005;46:ARVO E-Abstract 2183), it may alter the relative proportion of fibrillar collagen present in the mid- and posterior stromal layers and explain the lack of detection of any preferred orientation in the mid-stroma of these corneas.

Figure 2B shows the collagen fibril orientation in the middle layer of cornea P2 (200–400 μm from the corneal surface). Collagen fibrils were preferentially aligned in orthogonal directions throughout the 7.5-mm corneal disc and the amount of preferentially aligned collagen appeared to increase toward the periphery.

Figure 2C shows the predominant orientation of collagen in the posterior 200 μm of cornea P2 and in its full thickness scleral rim. The relative size of the polar plots (as shown by the color key) is greater in this layer than in the anterior and middle layers, indicating proportionally more aligned collagen in this region of the tissue. The collagen arrangement seen in the central 8 to 9 mm of the posterior cornea was similar to that found in previous x-ray scattering studies of full-thickness corneas.^{8,10,12,18,19} Consistent with previous studies of whole corneas, the orthogonal orientation of collagen fibrils in the

central cornea was gradually replaced with a tangential arrangement near the limbus and the amount of aligned collagen increased with distance from the center.^{4,8,15,19}

Figure 3 shows the distribution of collagen fibrils in the anterior, middle, and posterior layers of cornea P2. Figures 3A, 3C, and 3E all indicate that the total scatter from fibrillar collagen remained fairly constant in the central 3 mm of the cornea (yellow/orange contours) but increased toward the periphery (red/black contours). This finding was expected, as all three layers were of a similar tissue thickness (200 μm). The increase in total collagen content observed in the posterior stromal layer, beyond the region from which the anterior and mid stromal discs were removed (Fig. 3E), was the result of x-ray scattering data being gathered at full tissue thickness.

Scatter from preferentially aligned collagen in the anterior, middle, and posterior layers (cornea P2) is shown in Figures 3B, 3D, and 3F. The anterior layer has less aligned collagen than the middle layer, which in turn has less than the posterior layer, as indicated by the colors of the contours. In the posterior layer (Fig. 3F), the diamond shape seen in normal full-thickness corneas^{8,19} can also be seen here encroaching on the central region, particularly in the top right quadrant at the 2 o'clock position. This feature has been postulated to arise from a system of anchoring lamellae that traverse the peripheral cornea, and it would seem from this result that these lamellae are located in the posterior third of the cornea, although further research will be needed to confirm this.

Table 2 shows the proportion of collagen lamellae (within the plane of the stroma) that are preferentially oriented (over and above a population of collagen fibrils oriented equally in all directions), in the central 7 mm of the anterior, middle, and posterior regions of three corneas of comparable thickness (B3, P1, and P2). The proportion of aligned collagen at each tissue depth was calculated by dividing the intensity of x-ray scatter from preferentially aligned collagen at each sample point by the total x-ray scatter intensity at that point (expressed as a percentage) and then averaging the calculated values over the central 7-mm region. It is clear that the proportion of preferentially oriented collagen increases throughout the thickness of the tissue.

DISCUSSION

For the first time, the predominant orientation and relative mass distribution of collagen at different depths throughout the human cornea has been revealed. The results presented in this article indicate that the collagen fibrils are proportionally more aligned in the posterior two thirds of the cornea whereas in the anterior third, the orientation of collagen is more isotropic.

Most previous x-ray scattering studies of corneal collagen orientation have been performed on full-thickness corneas.^{8,19–21} Electron microscopy, on the other hand, has provided information on the arrangement of the collagen fibrils at different corneal depths, but it has not been so successful in determining larger scale orientation¹¹; furthermore, considerable variation appears to exist between studies. This variation has been attributed to methodological factors, mainly tissue preparation.^{22–24} With the evolution of femtosecond laser technology, a human cornea can now be delaminated into separate uniform layers of predetermined thicknesses with minimal tissue alteration.^{25–27} Using this method to accurately separate the cornea into anterior, middle, and posterior layers, and x-ray scattering techniques to determine the precise orientation of collagen and its relative mass distribution at specific points within each layer, we have been able to determine the gross organization of collagen fibrils throughout the thickness of the human cornea.

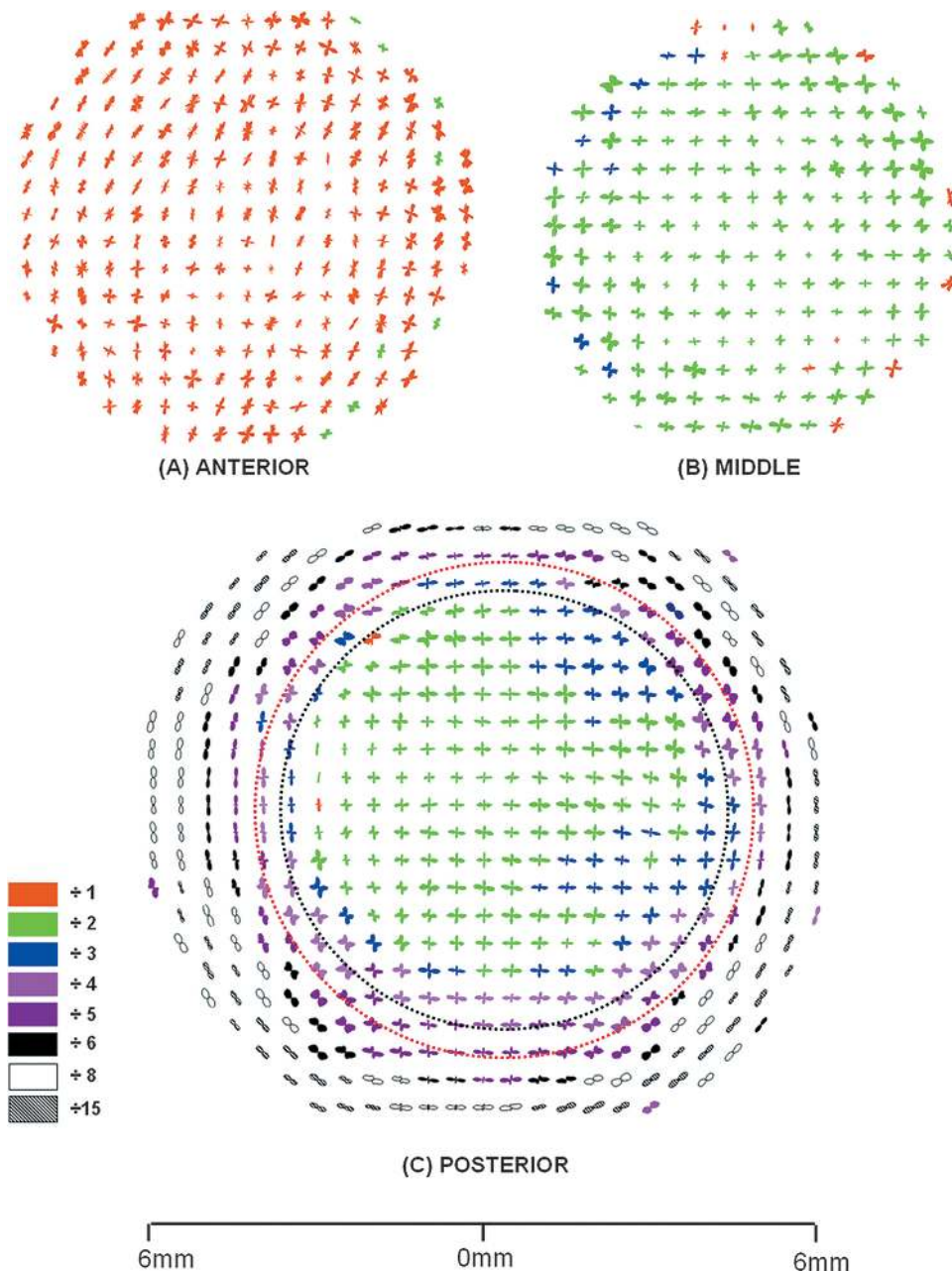


FIGURE 2. Polar plot maps showing collagen fibril orientation at 0.5-mm intervals across the anterior third (A) and middle 200 μm region (B) of a human cornea (P2). The posterior 200 μm of the cornea (with a full-thickness scleral rim) (C), is shown after removal of the anterior (red dotted line) and middle (black dotted line) layers from the central 8- to 9-mm region. Polar plots have been scaled down to fit onto the grid as indicated in the color key.

Various studies have reported that regional differences in the arrangement of collagen lamellae exist across the cornea. Kokott⁹ was the first to suggest that collagen fibrils in the deeper layers of the central cornea lie mainly in the superior-inferior and nasal-temporal meridians. X-ray scattering studies later confirmed this observation, and indicated that this preferred orientation was more prevalent in the posterior half of the cornea.¹⁰ Analysis of low-angle x-ray scattering patterns revealed that, in total, around two thirds of stromal collagen fibrils lie within 45° of the superior-inferior and nasal-temporal directions¹² and recently, Boote et al.¹⁸ has shown that, on average, these oriented fibrils lie in the same quantity along the superior-inferior and nasal-temporal meridians. The predominantly orthogonal arrangement of collagen seen in the central cornea is gradually lost toward the periphery, as fibrils adopt a more tangential orientation (with respect to the edge of the cornea). This gradual change in collagen orientation across the cornea concludes with a circumferential annulus of collagen at

the limbus.^{4,9,15} Transmission and scanning electron microscopy studies showed that collagen lamellae in the anterior one third of the stroma are narrower and more irregularly interwoven than are those in the deeper two thirds of the cornea.^{1,11,28-29} Many of these anterior lamellae insert into Bowman's layer, as has been elegantly demonstrated using second harmonic imaging confocal microscopy.³⁰

The results of the present study are in agreement with previous studies and show an isotropic orientation of collagen fibrils in the first 200 μm of the cornea. Previous depth studies have been limited to the central cornea,^{10,30} whereas in this study, both the central and paracentral regions of the cornea were examined. However, due to the maximum 9-mm cut diameter of the femtosecond laser, it was not possible to determine the collagen fibril orientation as a function of tissue depth around the limbus. As shown in Table 1, cuts were made at different tissue depths in each cornea to pinpoint the precise location at which the observed orthogonal preferred orienta-

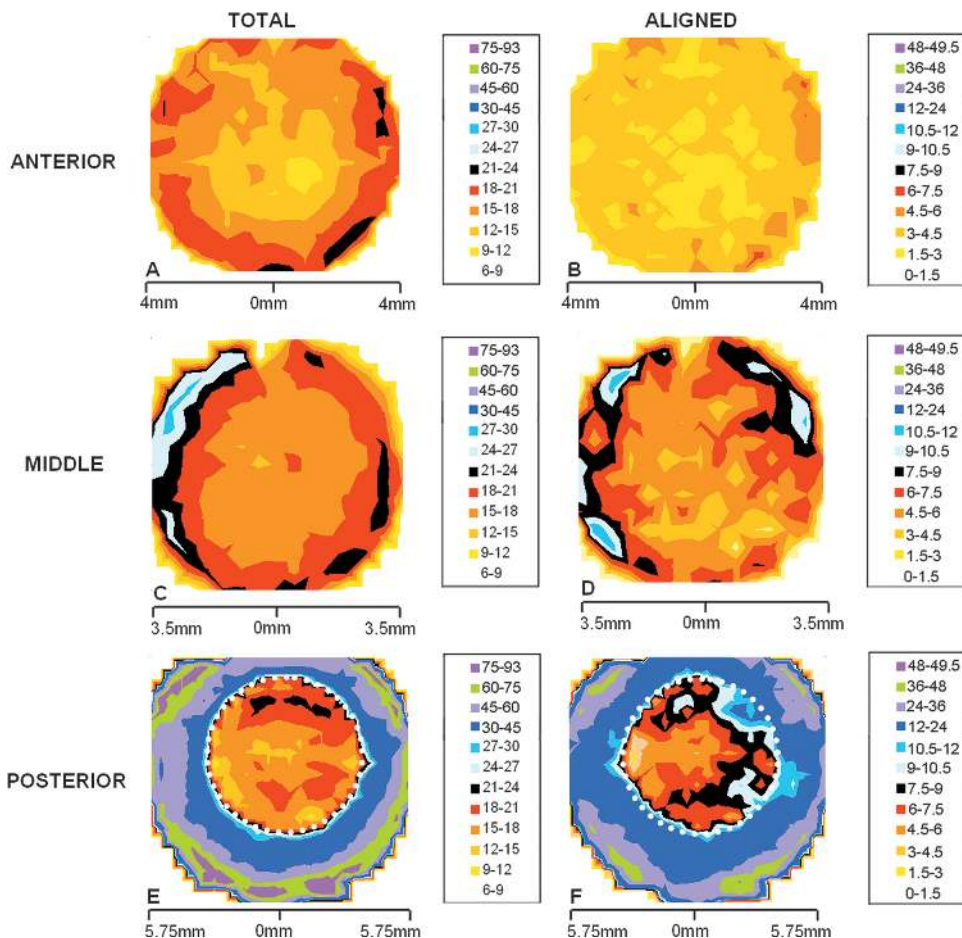


FIGURE 3. Contour maps showing the intensity of x-ray scatter (arbitrary units) from total fibrillar collagen (A, C, E) and preferentially aligned collagen (B, D, F) in the anterior (A, B) and middle (C, D) stroma of cornea P2 and in the remaining posterior cornea with scleral rim (E, F). *Dotted white line:* The central 8- to 9-mm region of the cornea from which the anterior and middle layers were removed.

tion of collagen predominates. Analysis of the x-ray scattering data showed that this arrangement of collagen began beyond the anterior third of the cornea.

The irregular fibril orientation in the anterior stroma was most pronounced in the central 4 mm area. Beyond that, some lamellae were found to be aligned in orthogonal directions, as demonstrated by the presence of cross-shaped plots toward the periphery in Figure 2. More orthogonally oriented lamellae also occurred away from the center in the middle stroma (Fig. 2B) as evidenced by the increase in the size of the plots. Since the femtosecond laser's mode of operation allows cuts to be achieved at predetermined depths with an accuracy of $\pm 14 \mu\text{m}^{25}$ and the damage to surrounding tissues is minimal,²⁶ no significant changes to collagen lamellae are likely to have been induced a result of the isolation process. The observed increase in collagen scatter toward the peripheral cornea is therefore unlikely to have been caused by differences in section thickness and was probably caused by changes in collagen density or hydration, both of which affect x-ray scatter intensity.

TABLE 2. The Percentage of the Total Collagen Fibrils That Were Preferentially Aligned in the Anterior, Middle, and Posterior Layers of Three Human Corneas

Layer	Cornea			Average
	B3	P1	P2	
Anterior	24	22	21	22
Middle	30	32	31	31
Posterior	41	45	41	42

In addition to polar plots, contour maps showing the relative distribution of aligned collagen mass quantitatively support the finding that the anterior third of the cornea is less aligned collagen than the posterior two thirds. This finding is further illustrated when the amount of preferentially aligned collagen in each layer is shown as a percentage of the total collagen (Table 2). In the posterior stroma, 42% of the lamellae are preferentially aligned in the vertical or horizontal direction (~21% in each direction), leaving 58% isotropically arranged. This means that, in the posterior stroma, the vertical and horizontal directions are reinforced significantly compared with other directions. In the anterior stroma, this value drops to 11% in each direction. Biomechanical strip testing has revealed that full thickness strips cut from the vertical and horizontal directions of human corneas are between 13% and 25% stronger than strips from intermediate directions.³¹ Our results suggest that the additional strength comes from lamellae in the posterior corneal stroma.

A further interesting observation made in this study was that the skewed diamond-shaped arrangement seen previously in contour maps of aligned collagen distribution, which is thought to be due to the presence of additional lamellae that traverse the peripheral cornea,^{8,19} seems to occur only in the posterior region of the cornea. The posterior third of the cornea clearly has an interesting, nonisotropic, lamellar organization that may extend into the peripheral region and the limbus and thus warrants further investigation.

Current popular refractive surgeries involve the ablation of corneal lamellae. These surgeries have been improved using a fairly empiric approach and complex assumptions, and as a result, the outcomes of refractive surgery may, and sometimes

do, differ from those expected. Part of this results from a limited understanding of the organization of collagen fibrils in the cornea³² and part because of similar problems with wound healing. Currently, finite element models are being developed to simulate the biomechanical response of the cornea, in an attempt to more accurately predict the result of refractive surgery in terms of shape and astigmatism. It is believed that the quantitative information presented herein may be helpful for improving understanding of the biomechanical properties of the cornea and may add further details about collagen fibril organization to computational finite element models.^{33,34}

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