3D collagen orientation study in human cornea using x-ray diffraction and femtosecond laser technology

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Abstract

**Aim:** To study the distribution and predominant orientations of Type 1 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 corneal lamellae preferred orientations.

**Methods:** Femtosecond laser technology was used to cut five human corneas into separate layers with predetermined thicknesses, and wide-angle x-ray diffraction was used to study the gross collagen fibril orientation and distribution within each layer at 0.5mm intervals.

**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 towards the periphery. However, the anterior cornea (33% of total corneal thickness) showed no systematic preferred lamella orientations.

**Conclusion:** In the posterior two-thirds of the human cornea, collagen lies predominantly in the vertical and horizontal meridians (directed towards the four major rectus muscles), whereas collagen in the anterior third of the cornea lies more equally in all directions. The predominantly orthogonal arrangement of collagen in the mid- and posterior stroma may help to reinforce the cornea by a factor of up to 21% in each direction, allowing it to withstand the pull of the extraocular muscles, whereas the more isotropic arrangement in the anterior
cornea is believed to play an important role in the biomechanics of the cornea by supporting the anterior corneal curvature.
**Introduction**

Corneal transparency depends on the unique architecture of the stroma, which accounts for 90% of the corneal thickness. In humans, the central stroma is made of ~200 stacked lamellae that lie essentially parallel to the corneal surface (Maurice 1957). These lamellae do not generally have the same orientation as each other; the change in orientation between lamellae may be any angle between 0 and 180° (Maurice 1969; Radner et al. 1998; Newton and Meek 1998b). 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 (Quantock et al. 2001; Meek et al. 2003). 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 (Farrell and McCally 2000; Aghamohammadzadeh et al. 2004).

In the literature, many studies have been done to find out the collagen fibril orientation within the human cornea (Kokott 1938; Meek et al. 1987; Komai and Ushiki 1991; Daxer and Fratzl 1997; Aghamohammadzadeh et al. 2004; Misson, 2007). The main outcomes of 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 centre of the cornea and is maintained to within 2mm of the limbus, where a gradual change to a tangential disposition occurs. However, the collagen fibril orientation and distribution at
different depths of the human cornea are still not well investigated. Therefore, in
the present paper, femtosecond laser technology was used to cut the human
corneas into different depth layers and wide-angle x-ray diffraction was used to
study the collagen fibril orientation and distribution within each layer. We regard
this as the first step towards a full three-dimensional reconstruction of corneal
lamellae preferred orientations. The results of this study should help us elucidate
the biomechanical reasons for some refractive problems, such as post-cataract
astigmatism or post-LASIK ectasia.

Materials and Methods

Five human corneas with a rim of sclera were used in this study. Three of them
(C1, C2 and C3) were obtained from Bristol Eye Bank (Bristol, UK); they were
immersed into 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) and kept at 37°C. The remaining two corneas
(OS and OD) were obtained from a pair of donor globes provided by the National
Disease Research Interchange (Philadelphia, USA) which were transported to
Cardiff University on dry ice. Prior to experimental use the globes were allowed
to thaw at room temperature and the corneas removed. Due to post-mortem and
storage change, some of the corneas had become swollen. Using a Pachette2™
Ultrasonic Pachymeter (DGH Technology, USA), the measured corneal thickness
of the five corneas ranged from 570 µm to 1040µm. However, we have
previously shown that even severe swelling does not change the directions of
corneal lamellae within the plane of the cornea (Meek et al., 2005a). All corneas were stored in 4% paraformaldehyde at 4°C until the experiment was carried out.

On the day of the experiment, each cornea was placed in a sterile Barron artificial anterior chamber (Katena Products Inc., NJ, USA) as shown in Figure 1, which was originally designed to safely facilitate the positioning, securing and inflating of donor corneas during corneal transplant and lamellar surgery procedures. In this study, the purpose was to retain corneal shape and curvature during the experiment by means of pumping a balanced salt solution underneath the cornea via the two tube ports with lockers.

**Figure 1:** The Barron Artificial Anterior Chamber. This figure shows the human cornea (yellow arrow) placed over the tissue retainer while the scleral annulus is located over the ridge; and clamped by the locking ring. A small diameter Petri dish was trephined centrally and attached firmly to the Barron chamber by silicon glue in order to work as a liquid reservoir to keep the cornea hydrated.
Three corneal lamellar cuts were then created using a femtosecond laser (IntraLase Corp., Irvine, CA), as shown in Figures 2, and 3. Cut diameter, cut depth, and other cut parameters are shown in Table 1.

Figure 2: (A) Intralase femtosecond laser system. (B) The position of the Barron chamber with the human cornea during corneal lamellar cuts. Note the patient interface (highlighted by a red arrow) that is attached to the laser machine and used to flatten the cornea and through which the laser beam passes.
Figure 3: Schematic diagram showing the way the cornea was cut into three layers. The thickness of each layer (anterior, middle and posterior) varied between corneas.

Due to the limited availability of the IntraLase femtosecond laser machine, the experiments were conducted at St. Thomas’ Hospital (London, UK) and at the Centre for Sight (East Grinstead, UK). The femtosecond laser cut was in a raster pattern without a hinge for all corneas, as shown in Table 1. Different layer thicknesses were produced in different corneas in order to provide a range of sampling regions throughout the stromal depth, these are shown in Table 2. The first cut layer (the anterior layer) was 8.5 mm in diameter and the second, middle layer measured 7.5mm. Each layer was carefully removed from the cornea using tweezers and the 12 o’clock position marked by means of a small incision. The individual layers were kept separately in a fresh 4% paraformaldehyde solution.

The remaining part of the cornea (the posterior layer) varied in thickness between samples and ranged from about 200µm to 800µm, as measured by ultrasonic pachymetry. The corneoscleral button was dissected using a 12mm trephine (Altomed, UK) in order to remove unwanted scleral tissue. It was then put in 4% paraformaldehyde until the time of the x-ray diffraction experiment at the
Synchrotron Radiation Source (Daresbury, UK), where collagen fibril organisation was mapped in detail in each layer.

<table>
<thead>
<tr>
<th>Method</th>
<th>Raster</th>
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<tbody>
<tr>
<td>Diameter</td>
<td>7.5mm to 8.5mm</td>
</tr>
<tr>
<td>Depth</td>
<td>120µm to 300µm</td>
</tr>
<tr>
<td>Hinge</td>
<td>No</td>
</tr>
<tr>
<td>Hinge Angle</td>
<td>No</td>
</tr>
<tr>
<td>Cut Energy</td>
<td>0.80 µJ</td>
</tr>
<tr>
<td>Side Cut Angle</td>
<td>70°</td>
</tr>
<tr>
<td>Pocket Start Depth</td>
<td>220 µm</td>
</tr>
<tr>
<td>Spot/Line Separation</td>
<td>4x4 µm</td>
</tr>
</tbody>
</table>

**Table 1:** Parameters of IntraLase femtosecond laser machine. Different depths and diameters were used to cut the cornea (listed in Table 2).

**X-Ray Diffraction**

In order to minimise tissue dehydration during X-ray exposure, each piece of corneal tissue was wrapped in Clingfilm™ (Superdrug Stores Plc., Croydon, UK) and placed in an airtight Perspex (Databank, UK) chamber with Mylar (Dupont-Teijin, UK) windows, and sealed with grease. All experiments were performed at station 14.1 at the UK Synchrotron Radiation Source, using a wide angle beam
with a wavelength of 0.1488 nm wavelength and a 0.2 mm x 0.2 mm square cross-section at 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 the experiment. Differences in x-ray exposure time were normalised for during data analysis. X-ray scatter patterns were collected at 0.5mm intervals over each corneal layer and were recorded on a Quantum 4R Charge-Couple Device (CCD) detector (ADSC, Poway, CA) located 150 mm behind the specimen. A lead beamstop was positioned between the sample and the detector to stop any undeviated x-rays.

All data were then transferred to the Structural Biophysics Laboratory at the School of Optometry and Vision Sciences (Cardiff University) for analysis. Each x-ray scatter intensity profile was normalised against variations in x-ray exposure time and beam intensity. Figure 4 shows the major steps of X-ray diffraction analysis. A high angle diffraction pattern from the central human cornea (Figure 4A) shows the beamstop position in the centre and a ring of x-ray scatter corresponding to equatorial (i.e. perpendicular to the fibril axis) x-ray scattering from the collagen molecules. Four lobes (reflections) of increased x-ray scatter intensity can also be seen which 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° (Holmes et al. 2001), so we can use the molecular alignment as a good indication of the fibril alignment. The radial “background” scatter (arising from non-collagenous components in the cornea) was subtracted from the
reflection by taking 256 segments and fitting a power law function radially outward from the centre of each x-ray scatter pattern (Newton and Meek 1998a; Newton and Meek 1998b). Following this, the scatter was summed for each radial position and the summed x-ray scatter intensity plotted as a function of angle (Figure 4B). This x-ray scatter distribution was divided into two components, as shown in Figure 4B: (A) isotropic scatter from collagen fibrils distributed equally in all directions (i.e. randomly orientated), and (B) preferentially aligned fibril scatter. The isotropic scatter was subtracted (Daxer and Fratzl 1997; Boote et al. 2004), and after shifting the data by 90° to account for the fact that the reflections are at right angles to the fibril axes (Figure 4C), the preferentially aligned fibril distribution was plotted in polar coordinates as seen in Figure 4D (Connon and Meek 2003). Displaying Figure 4C as a polar plot (Figure 4D) allows us to readily see the preferred fibril directions that gave rise to the X-ray reflection in Figure 4A. The radial size of the plot in a given direction is proportional to the number of fibrils disposed in that direction.
Figure 4: Main steps of XRD analysis. (A): A high angle XRD pattern recorded from the central human cornea. The x-ray reflection is comprised of four lobes. (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 orientated collagen (shaded area). (C): Distribution of scatter intensity after the subtraction of isotropic scatter and a 90 degree phase shift. Introducing the phase shift effectively converts Figure 4B into a plot of relative fibril number versus angle. (D): Figure C plotted in polar coordinates.

One point in the x-ray pattern is arrowed in A and arrows in B-D allow this point to be followed through each stage of the analysis.

Each X-ray pattern was analysed to produce a polar plot representing quantitatively the amount of collagen preferentially aligned in a given direction or
directions, summed throughout the full thickness of the tissue at that point. By recording x-ray patterns across the whole tissue, an assembly of polar plots was then created in order to visualise the spatial distribution of preferentially aligned collagen fibrils.

The total area under the scatter intensity graph in Figure 4B is proportional to the total amount of collagen at that point in the tissue. This can be subdivided into the amount of isotropic collagen (shaded area) and the amount of preferentially aligned collagen (clear area). By calculating these quantities at each point across the tissue, maps showing the relative distribution of total and aligned collagen fibrils over the whole tissue were produced.

Results

Table 2 shows the thickness of each corneal layer that was cut by the femtosecond laser.

<table>
<thead>
<tr>
<th>Cornea</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>OD</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer (µm)</td>
<td>120 (12%)</td>
<td>140 (14%)</td>
<td>160 (28%)</td>
<td>250 (36%)</td>
<td>200 (33%)</td>
</tr>
<tr>
<td>Middle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer (µm)</td>
<td>120 (12%)</td>
<td>140 (14%)</td>
<td>160 (28%)</td>
<td>250 (36%)</td>
<td>200 (33%)</td>
</tr>
<tr>
<td>Posterior (remaining)</td>
<td>800 (76%)</td>
<td>710 (72%)</td>
<td>250 (44%)</td>
<td>200 (28%)</td>
<td>200 (33%)</td>
</tr>
<tr>
<td>Total Thickness</td>
<td>1040</td>
<td>990</td>
<td>570</td>
<td>700</td>
<td>600</td>
</tr>
</tbody>
</table>
Table 2: The thickness (in micrometres) of the anterior and middle layers that was cut by femtosecond laser. Numbers in brackets represent the percentage of the total corneal thickness. The posterior (remaining) layer thickness was measured by ultrasonic pachymetry. Note that the C3, OD and OS corneas were only slightly swollen compared to C1 and C2, which were swollen to almost twice physiological thickness.

Figure 5A shows the orientation of collagen fibrils in the anterior one third of cornea OS. No consistent preferred orientation can be detected in this anterior region (approximately 33% of total corneal thickness) except, perhaps, towards the periphery. The other corneas showed similar results in the anterior layers; C1 and C2 showed the same result in the middle layers also.

Figure 5B shows the collagen fibril orientation in the middle of cornea OS, that is, the second 200 µm cut. Collagen fibrils appear well oriented orthogonally all over the 8mm corneal tissue with an increase in the collagen density towards the periphery.

Figure 5C shows the collagen fibril orientation in the remaining corneal tissue with part of sclera. The collagen fibrils appear highly orientated over the entire cornea including the remaining 200µm centrally. The collagen arrangement in the central 8-9 mm is similar to that found in the centre of a full thickness normal human cornea and is consistent with previous wide-angle XRD work (Meek et al. 1987; Daxer and Fratzl 1997; Aghamohammadzadeh et al. 2004; Boote et al. 2005; Boote et al. 2006). The orthogonal orientation of the central corneal fibrils is shifted to a tangential arrangement near the limbus with a higher proportion of
aligned collagen similar to that reported previously (Newton and Meek 1998a; Newton and Meek 1998b; Aghamohammadzadeh et al. 2004; Boote et al. 2006).

**Figure 5:** Polar plot maps showing collagen fibril orientation at 0.5mm intervals in the anterior third (A) and middle 200µm region (B) of a human cornea (OS). The posterior 200µm of the cornea (with a full thickness scleral rim) (C), is shown
following removal of the anterior (red dotted line) and middle (black dotted line) layers from the central 8-9mm region. Polar plots have been scaled down to fit onto the grid as indicated in the colour key.

Figure 6 (A-F) shows the distribution of collagen fibrils in the anterior, middle, and posterior (remaining) corneal layers. Figures 6 A, C and E all indicate that the total scatter from fibrillar collagen remains fairly constant in the central 3mm (orange contours) but increases towards the periphery (red/black contours). This is expected as the thickness of all three layers in cornea OS was approximately the same (200µm). However, the posterior total map (Figure 6E) represents the collagen distribution in the remaining cornea after the anterior and middle central layers were removed and the collagen distribution in the central area of this plot is less uniform than in the anterior and middle layers (Figs 6 A and C). There is obviously a rapid increase in collagen content outside the region from which the anterior and middle sections were cut, as seen by the blue contours.

Scatter from preferentially aligned collagen for the anterior, middle and posterior layers (cornea OS) is shown in Figures 6 B, D and F. The anterior layer has less aligned collagen than the middle layer, which in turn has less than the posterior layer, as indicated by the colours of the contours. In the posterior layer (6F), the diamond shape that is seen in a normal cornea (Aghamohammadzadeh et al. 2004; Boote et al. 2006) can also be seen here encroaching into the central region, particularly in the top right-hand quadrant at about the 2 o’clock position. This feature has been postulated to arise from a system of anchoring lamellae,
and it would seem from this result that these lamellae are located in the posterior third of the cornea, although further research is needed to confirm this.

Figure 6. 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 third (A and B), the middle 200 µm (C and D) and the remaining posterior cornea with scleral rim (E and F). The dotted white line in E and F represents the central 8-9mm region of the cornea from which the anterior and middle layers were removed.
Table 3 shows the proportion of collagen lamellae (within the plane of the stroma) that are orientated in a particular direction (over and above a population of collagen fibrils orientated equally in all directions) in the central 7mm of the anterior, middle and posterior regions of three corneas of comparable thickness (C3, OD and OS). The proportion of aligned collagen at each tissue depth was calculated by dividing the intensity of 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 7mm region. It is clear that the proportion of preferentially orientated collagen increases throughout the thickness of the tissue.

<table>
<thead>
<tr>
<th>Cornea Layer</th>
<th>C3</th>
<th>OD</th>
<th>OS</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>24%</td>
<td>22%</td>
<td>21%</td>
<td>22%</td>
</tr>
<tr>
<td>Middle</td>
<td>30%</td>
<td>32%</td>
<td>31%</td>
<td>31%</td>
</tr>
<tr>
<td>Posterior</td>
<td>41%</td>
<td>45%</td>
<td>41%</td>
<td>42%</td>
</tr>
</tbody>
</table>

**Table 3:** The percentage of the total collagen fibrils that are preferentially aligned in each layer of three human corneas together with the average values.

**Discussion**

By using a femtosecond laser and x-ray scattering, for the first time, a whole map for the collagen fibril orientation and distribution at different human corneal
depths has been shown. The femtosecond laser is particularly suitable for this kind of study as it is a low energy method that causes minimal tissue alterations (Kezirian and Stonecipher, 2004; Sarayba et al., 2007). The results indicate that the collagen fibrils are highly oriented in the posterior two thirds of the corneal thickness whereas the anterior third reveals no specific collagen arrangement.

Most results of previous x-ray scattering studies examining collagen orientation were acquired from full thickness corneas (Meek and Quantock 2001; Meek et al. 2005b). Electron microscopy, on the other hand, has provided much information on the arrangement of the collagen fibrils at different corneal depths, but it has not been so successful in determining larger scale orientation (Komai and Ushiki 1991); furthermore, it has shown considerable variation among studies. This variation has been attributed to methodological factors, mainly tissue preparation (Freund et al. 1995; Daxer et al. 1998; Meek and Fullwood 2001). With the revolution of femtosecond laser technology, a human cornea can now be cut into separate uniform layers with predetermined thicknesses and, using this method, we have therefore been able to determine the gross collagen fibril organisation from each layer using x-ray diffraction.

Previous corneal studies reported that the arrangement of the collagen lamellae shows regional differences. Kokott was the first to suggest that collagen fibrils in the deeper layers of the central cornea take on a preferential orientation along the superior-inferior and nasal-temporal corneal meridia (Kokott 1938). X-ray scattering studies later confirmed this, and suggested that the preferred
orientation is more prevalent in the posterior half of the cornea (Meek et al. 1987). Further quantitative analysis by low-angle XRD has indicated that, in total, around two thirds of the fibrils of the stroma tend to lie within the 45° sectors of the superior-inferior and nasal-temporal directions (Daxer and Fratzl 1997). Recently, Boote et al. (2005) found that, on average, these oriented fibrils lie in the same quantity along the superior-inferior and nasal-temporal meridians. This arrangement no longer predominates at the periphery of the cornea, where fibrils tend to take the shape of a circumferential annulus at the limbus (Schwalbe, 1870; Kokott, 1938; Newton and Meek 1998a; 1998b). 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 (Hogan et al. 1971; Davson 1984; Klyce and Beuerman 1989; Komai and Ushiki 1991). Many of these anterior lamellae insert into Bowman’s layer, as was elegantly demonstrated using second harmonic imaging confocal microscopy (Morishige et al. 2007).

The results herein are in agreement with the previous studies and show that the collagen fibril orientation in the first third of the corneal thickness, that is, 200µm out of 600 µm is irregular whereas the remaining deeper layers show a preferred orthogonal orientation. Furthermore, the results cover the central and paracentral cornea, that is, 8-9mm diameter, whereas previous depth studies were limited to the centre of the cornea (Meek et al., 1987; Morishige et al. 2007). Due to the limitation of a femtosecond laser to a maximum of 9mm diameter cut, it was difficult to show the collagen fibril orientation around the limbus regions.
Table 2 shows that cuts of different thickness have been used with each cornea in order to try to pinpoint the start of the preferred orthogonal fibril orientation; this has been found to occur after the first third of corneal total thickness.

The irregular fibril orientation in the anterior stroma was more pronounced in the central 4mm area. Beyond that, some lamellae were found to be orthogonal, as demonstrated by the presence of cross-shaped plots towards the periphery in Figure 5. More orthogonal lamellae also occur away from the centre in the middle stroma (Figure 5B) as evidenced by the increase in the size of the plots. The femtosecond laser can cut a section of uniform thickness to a tolerance of a few percent, so the increase in collagen scatter peripherally is unlikely to be due to differences in section thickness. They are more likely to be caused collagen density or hydration changes towards the peripheral cornea, both of which can affect the x-ray scatter intensity.

In addition to polar plots, contour maps (Figures 6 B, D and F) quantitatively support the finding that the anterior one third of corneal thickness has less aligned collagen than the posterior two thirds. The percentage of preferentially aligned fibrils in each layer shows that there is an increase in fibril alignment from the anterior towards the posterior layer (Table 3). In the posterior stroma, 42% of the lamellae are preferentially vertical or horizontal (approximately 21% in each direction), leaving 58% isotropically arranged. This means that, in the posterior stroma, the vertical and horizontal directions are reinforced significantly compared to 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 (ElSheikh et al, 2008). Our results suggest that the additional strength comes from lamellae in the posterior corneal stroma.

An interesting observation was also made here that the diamond shape seen in the distribution of preferentially aligned collagen in the normal cornea (Aghamohammadzadeh et al. 2004; Boote et al. 2006) seems to occur in the posterior layer after removal of the anterior two-thirds. The posterior third clearly has an interesting, non-random, lamellar organisation across the cornea 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 empirical approach and complex assumptions. Therefore, the outcomes of refractive surgery may differ from the expected ones, largely because of the lack of information available on the organisation of collagen fibrils in the cornea (Fullwood 2004). Currently, finite element models are being developed to simulate the biomechanical response of the cornea and predict the result of refractive surgery in terms of shape and astigmatism. It is believed that the quantitative information presented herein is essential for an understanding of the biomechanical properties of the cornea and may add crucial details about the collagen fibril organisation to the ongoing
computational finite element models (Pinsky et al. 2005; Lanchares et al. 2008) and, hence, help in the geometric interpretation of the corneal and surgical data. For example, the biomechanical changes that are caused by flap cutting and laser ablation in LASIK surgery could be predicted preoperatively once a full quantitative three-dimensional reconstruction of corneal lamellae preferred orientations is available. The data presented herein is the first step towards such a reconstruction.

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