Evolution of the vertebrate corneal stroma

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Abstract

Although the cornea is the major refractive element of the eye, the mechanisms controlling corneal shape and hence visual acuity remain unknown. To begin to address this question we have used multiphoton, non-linear optical microscopy to image second harmonic generated signals (SHG) from collagen to characterize the evolutionary and structural changes that occur in the collagen architecture of the corneal stroma. Our studies show that there is a progression in complexity of the stromal collagen organization from lower (fish and amphibians) to higher (birds and mammals) vertebrates, leading to increasing tissue stiffness that may control shape. In boney and cartilaginous fish, the cornea is composed of orthogonally arranged, rotating collagen sheets that extend from limbus to limbus with little or no interaction between adjacent sheets, a structural paradigm analogous to 'plywood'. In amphibians and reptiles, these sheets are broken down into broader lamellae that begin to show branching and anastomosing with adjacent lamellae, albeit maintaining their orthogonal, rotational organization. This paradigm is most complex in birds, which show the highest degree of lamellar branching and anastomosing, forming a 'chicken wire' like pattern most prominent in the midstroma. Mammals, on the other hand, diverged from the orthogonal, rotational organization and developed a random lamellar pattern with branching and anastomosing appearing highest in the anterior stroma, associated with higher mechanical stiffness compared to the posterior stroma.

Outline

Abbreviations

3D	Three dimensional
FFT	Fast Fourier Transform
HRMac	High Resolution Macroscopy
NLO	Non-linear optical
SHG	Second Harmonic generation

1. Introduction

Vision is arguably the most important sense to perceive our environment. While all the major phyla have structures that detect light information, their anatomy, origin and degree of sophistication differ immensely according to their environmental and functional constraints (Jonasova and Kozmik, 2008; Land and Nilsson, 2012). The evolution of visual perception has occurred in four stages: 1) simple photoreception; 2) photoreception with some degree of directionality; 3) low-resolution spatial vision and 4) high-resolution multipurpose vision (Land and Nilsson, 2012; Nilsson D-E, 2009). Comparative anatomical studies have identified a range of animal eyes, including pinhole eyes, reflecting mirror eyes and camera-type eyes with a single lens (Arendt and Wittbrodt, 2001; Lacalli, 2004; Land, 2012; Land and Nilsson, 2012).

In the vertebrate, camera-type eye, visual acuity is dependent, in part, on the crystalline lens and the transparent cornea, the eye's refractive components, which form an optical system that refracts and transmits light to the retina (Benedek, 1971; Clark, 2004; Delaye and Tardieu, 1983; Land and Nilsson, 2012; Piatigorsky, 2001; Ruberti and Klyce, 2002). The cornea is the tissue covering the front of the eye and it specifically fulfils two important roles. As the outermost layer of the eye, it helps to shield the internal parts of the eye and has a protective function dependent on the mechanical strength of the outer ocular tunic of which the cornea is a continuous part. The biomechanical strength of the cornea must therefore be great enough to prevent tissue rupture following blunt force trauma. These properties of the cornea are thought to be derived from the compositional makeup of the tissue, which is predominantly comprised of collagen fibrils and proteoglycans.

Aside from its protective role, the cornea also focuses light onto the retina and accounts for over 2/3 of the refractive power in the human eye. Corneal shape and curvature are vitally important to determining the refractive power and aberrations in corneal curvature can cause refractive errors including, myopia, hyperopia and astigmatism (Garner and Smith, 1997; Llorente et al., 2004; Mouroulis, 1999; Thibos, 2000). Overall, refractive

errors are the most common vision-related disorder, affecting over 200 million Americans (Wittenborn and Rein, 2013).

The refractive properties of the cornea are governed by Snell's law, also known as the law of refraction, which dictates that when light travels between two isotropic media, such as water, glass or air, the angle of refraction is proportional to the difference in refractive indices between the two media. Since the evolution of the vertebrate eye initially occurred in water, the cornea with a refractive index (1.376) almost identical to that of water (1.333) played little role in the refraction of light to the retina regardless of corneal shape, serving primarily as a transparent protective cover, while the crystalline lens with a higher refractive index (1.4-1.5) performed the refractive function (Collin and Collin, 2000; Leonard and Meek, 1997; Patel, 1987; Patel, Marshall and Fitzke, 1995; Sivak et al., 1989). The vertebrate cornea has a greater potential refractive power in air given the difference in the refractive index between air (1.000) and the cornea. Because of the large difference in these refractive indices, vertebrate eyes functioning in both environments would have to be hyperopic in water and myopic in air. To overcome this problem with excessive focusing power, adaptations in the corneal structure and curvature, as well as the accommodative ability of the lens, have emerged during evolution (Graham and Rosenblatt, 1970; Howland and Sivak, 1984; Knowles, Vollrath and Nishioka, 1967; Murphy et al., 1990; Sivak et al., 1989). A fine example of adaptations to an air/water visual existence is found among *Anableps anableps*, the "four-eyed" fish, where structural modifications in corneal shape and lens placement allow *Anableps* to simultaneously accommodate in both air and water (Schwab et al., 2001; Sivak 1976; Swamynathan et al., 2003). Similar to *Anableps anableps*, aquatic mammals like the seal also have a paracentral corneal region that is flat allowing them to have similar visual acuity in both air and in water (Land and Nilsson, 2012).

While the shape of the cornea determines its refractive power, the cellular, molecular and biomechanical mechanisms regulating shape have long served as one of the most intriguing questions in corneal biology that has remained largely unknown. The biomechanical properties of the cornea have been extensively examined by numerous investigators using distinct methodologies cornea has proven to be challenging and the

results reported in the literature vary from a few kilopascals to gigapascals, depending on the type of measurement and the experimental environment. This variation in corneal material properties has been attributed to the anisotropy in stromal architecture, particularly regarding collagen fibril organization that is thought to define the mechanical behaviour of the tissue (Martin and Boardman 1993; Martin and Ishida, 1989).

In this review, we focus on the structural and architectural differences in the collagen fiber/lamellar organization of the vertebrate cornea from different extant species that provide a range of corneal shapes and refractive adaptations that have been acquired during vertebrate eye evolution. To perform these structural analyses, we have used second harmonic generation (SHG) imaging to three-dimensionally reconstruct the collagen organisation and establish a 'structural blue-print' of the corneal stroma from these diverse corneal shapes. These studies have identified a common structural theme of increasing fiber/lamellar complexity involving branching and anastomosing of collagen bundles that appears to control regional corneal stiffness and, hence, corneal shape and biomechanics.

2. Imaging Corneal Stromal Structure

The corneal stroma represents 90% of the corneal thickness and is composed predominantly of fibrillar collagen representing 70% of the dry weight of the cornea (Abahussin et al. 2009). The basic structure of the stroma has been described using a wide range of different techniques (Abahussin et al. 2009, Aghamohammadzadeh, Newton, and Meek 2004, Daxer et al. 1998, Han, Giese, and Bille 2005, Komai and Ushiki 1991, Meek et al. 1987, Morishige et al. 2006), and has been shown to be comprised of uniformly thin (~32 nm diameter) collagen fibrils, which are bundled together to form collagen fibers or lamellae in a wide range of vertebrate corneas. The combination of electron microscopic studies, which provide insights on the corneal nanostructure by resolving individual collagen fibrils (Hamada et al., 1972; Komai and Ushiki 1991; Muller et al., 2001; Radner et al., 1998), and x-ray scattering studies which visualize the bulk collagen alignment across the entire cornea while measuring fibril diameter and spacing (Aghamohammadzadeh, Newton, and Meek 2004; Meek and Boote 2009), have provided the baseline of our current understanding of corneal

structure. The sum results of these studies indicate that collagen fibrils exist in bundles which coalesce to form approximately 200 lamellae organized parallel to the corneal surface with a bulk preferential alignment in the organization of the lamellae along the horizontal or vertical meridians of the cornea. In non-mammalian vertebrate corneas, each lamella is rotated about 90° relative to its adjacent lamella, acquiring an overall orthogonal arrangement (Aghamohammadzadeh, Meek and Boote 2009; Newton, and Meek 2004; Svoboda, 1991; Svoboda and Hay 1987; Svoboda et al., 1988; Trelstad and Coulombre, 1971). To the contrary, in mammals, collagen lamellae are randomly arranged in a single plane with large amounts of fiber branching and anastomosis, especially in the anterior corneal stroma (Morishige et al., 2006; Morishige et al., 2007; Winkler et al., 2011, Muller et al., 2001). Regarding collagen organization at the peripheral cornea, fibrils may exhibit a more circumferential orientation perhaps creating a boundary between corneal and scleral curvature (Aghamohammadzadeh, Newton, and Meek 2004; Kokott 1938).

While the cellular and molecular mechanisms involved in collagen fibrillogenesis are well established (Zhang et al., 2005), as well as the mechanisms underlying corneal transparency, considerably less is known concerning the mechanisms controlling corneal structure and biomechanics. Although the mechanical properties of the cornea have been comprehensively studied (Dupps et al., 2007; Hjortdal 1995, 1996; Hoeltzel et al., 1992; Hollman et al., 2002; Jue and Maurice, 1986; Last et al., 2012; Lepert et al., 2016; Liu and Roberts 2005; Mikula, Jester and Juhasz, 2016; Nyguist, 1968; Petsche et al., 2012; Scarcelli et al., 2015; Tanter et al., 2009; Woo et al., 1972; Zeng et al. 2001), the cellular and molecular mechanisms controlling these properties are not well understood. This challenge is mainly due to the difficulty in interpreting the anisotropy of the structural elements comprising the cornea in terms of their mechanical effects on determining tissue form and function. Amongst the various structural components, collagen certainly plays the fundamental role in defining the cornea's structural and biomechanical properties (Martin and Boardman 1993; Martin and Ishida 1989; Aghamohammadzadeh, Newton, and Meek 2004; Han, Giese, and Bille 2005; Ruberti and Zieske 2008). While fibrous collagen exhibits distinct longitudinal tensile strength, it is comparatively weak along the other axes. Therefore, it is important to appreciate at

this point, that the unique spatial orientation and supramolecular architecture of collagen fiber most likely will have a major impact on the mechanical properties of tissues, as well as exert distinct effects on the corneas response to mechanical strains that will define the shape and refractive power of the cornea. This understanding was first articulated by Kokott in 1938 when he attempted to map the supramolecular organization of collagen in the cornea and sclera in order to develop a structural 'blue-print' of the eye to identify the mechanical mechanisms controlling ocular shape (Kokott, 1938). While his pioneering studies were the first to address this question, improvements in optical and digital imaging now enable the spatial mapping of collagen over large regions of the eye to begin to build-up a true collagen 'blue-print' on which mechanical models can be developed that can lead to a better understanding of how corneal shape is controlled.

2.1. SHG imaging of Collagen

SHG is a non-invasive, absorption-free non-linear process, wherein non-centrosymmetric materials have the capability of frequency doubling high intensity photonic radiation, which was first theoretically described by Goppert-Mayer in 1931, and later demonstrated by Franken et at in 1961 (Franken et al., 1961; Goppert-Mayer, 1931). SHG signals can be generated in biologic tissues by focusing very fast pulsed, infrared femtosecond laser light into tissues or cellular structures without central symmetry and reconstructing an image from the SHG signals collected from within the focal volume of the objective (Campagnola, 2011; Campagnola and Dong, 2009). The image is built point by point as the laser is focused to a new location in the sample and the SHG signal is collected. This happens in near real time due to the high speed of the laser scanning optics and the high computing power of modern computers. In the cornea, collagen molecules are the principal asymmetric structural molecules that generate SHG signals.

Since femtosecond laser pulses can generate extremely high field strengths when tightly focused within a tissue, they generate an oscillating polarization of the non-centrosymmetric oriented molecules that results in the frequency doubling of light to generate photons that are 1/2 the wavelength, leading to the generation of a visible light

photon when exciting with infrared laser light. Because these field strength conditions are only achieved within the focal volume of the femtosecond laser beam, imaging of structures can be performed non-invasively, deep within the tissue at high spacial and axial resolution (~1 μm), without the loss of signal that is introduced by the confocal pinhole. Importantly, SHG imaging is higly specific for fibrillar collagen which is non-centrosymetric along the fibrillar axis, yet centrosymetric when viewed in cross section (Campagnola, 2011; Campagnola and Dong, 2009; Chen et al., 2012; Williams, Zipfel and Webb, 2005). Therefore, unlike electron microscopy (Figure 1A), SHG imaging detects only collagen bundles running in the same optical plane, while leaving undetected those fibers running out of plane as shown in (Figure 1B). Due to the high axial resolution of the SHG imaging process, it is possible to image collagen fibers at varying depths, creating virtual slices of the imaged tissue which can then be digitally combined and rendered as a three dimensional (3D) representation of the structure. It is therefore possible to reconstruct the 3D structure of collagen fibers over long distances, from millimetres to centimetres.

Since collagen is the most abundant protein in the body, providing the structural support to connective tissues including cornea, bone (Batge et al., 1992), tendon (Cen et al., 2008) and skin (Shoulders and Raines, 2009), SHG imaging has applications for characterizing the 3D structure of most if not all connective tissues (Campagnola et al., 2002; Chen et al., 2012; Strupler at al., 2007). While it cannot visualize individual collagen fibrils at the nanoscopic level, SHG does resolve higher levels of organization including bundles of fibrils or fibers and lamellae (Quantock et al., 2015).

A key advantage of SHG, compared to other imaging modalities, is that the tissue can be investigated non-invasively in its native state and over the last two decades, collagen SHG microscopy has emerged as a versatile and powerful tool to investigate collagen organization and tissue function. Alterations in fibrillar collagen structure and organization also play an important role in several disease states such as osteogenesis imperfecta and diabetes (Kowalczuk et al., 2013; Latour et al., 2012; Mostaço-Guidolin et al., 2013). Recent studies have also emphasized SHG's potential clinical application in early diagnosis of breast, ovarian and skin cancer, including discrimination between healthy

cells versus cancer cells and delineation of cancers of different stages (Conklin et al., 2011; Kirkpatrick, Brewer and Utzinger, 2007; Lin at al., 2006; Nadiarnykh et al., 2010; Provenzano et al., 2006; Sahai et al., 2005). As mentioned earlier, SHG imaging has opened up a new window through which the corneal stromal collagen organizational patterns can be visualized at the macroscopic level. Since the pivotal work by Hochheimer in 1982 who first reported the generation of SHG signals in the rabbit cornea (Hochheimer 1982), NLO SHG imaging rapidly found a place in investigative studies of the corneal collagen structure of various species including human (Aptel et al., 2010; Han et al., 2005; Morishige et al., 2006), mouse (Lo et al., 2006, Morishige et al., 2006), pig (Jay et al., 2008; Teng et al., 2006; Wang et al., 2008) and rabbit (Morishige et al., 2006). NLO SHG imaging has also facilitated the investigation of gross pathologic changes and matrix disorganization in pathological conditions such as keratoconus (Morishige et al., 2007; Tan et al., 2006), thermal injury (Lo et al., 2009; Tan et al., 2005), infectious keratitis (Tan et al., 2007), corneal oedema (Hsueh et al., 2009; Wu and Yeh, 2008), wound healing (Farid et al., 2008; Teng et al., 2007) and diabetes (Latour et al., 2012; Mostaço-Guidolin et al., 2013).

2.2. High Resolution Macroscopy (HRMac) SHG imaging

Although SHG imaging can be used to detect in-plane collagen fiber organization, the SHG signal is a function of the square root of the excitation power, and can only be generated in a very small focal volume, where the excitation laser beam intensity is high enough to cause nonlinear photon interactions. While this provides for submicron lateral and axial resolution, this severely limits the field of view to regions smaller than 0.2 mm^2, which is not compatible with tracking collagen fibers along the length of the cornea, particularly in very large vertebrates for which the corneal diameter may run many millimeters to centimeters. To greatly expand the field of view, we have used a technique called high-resolution macroscopy (HRMac) that combines high resolution SHG imaging with automated image acquisition and mosaic digital image reconstruction (Jester et al, 2010; Winkler et al, 2010). Using HRMac, large scale, 3D images of collagen fibers that extend across the entire cornea can be reconstructed, while maintaining high lateral and axial resolution. This provides for both micro- and macrostructural analysis of the collagen

organization generating digital images that can be 'zoomed' in and out, similar to what is obtained with 'Google® Maps'. These data sets can also be quantitatively evaluated for analysis of fiber complexity, size and orientation.

To generate HRMac images from the cornea, a vibratome was used to cut 150-300 µm tissue section of whole corneas, embedded in low melting point agar (Figure 2). Sections were then mounted on a glass coverslip holder and imaged using a laser scanning confocal microscope (Zeiss 510 LSM) with a femtosecond laser, tuned to 820 nm (Chameleon Laser, Coherent, Santa Clara, CA). High resolution, 3D image stacks were then collected over the entire vibratome section, from limbus to limbus. Image stacks were then concatenated to form a single image stack as shown in Figure 3 that would contain up to 80 Mega Pixel image planes covering 150-300 µm of corneal depth (Figure 4A). Since each HRMac image contains high resolution detail of in-plane collagen fibers, individual fibers can be semi automatically segmented in 3 dimensions (Figure 4B), and then extracted and viewed in different orientations (Figure 4C) to better view the complexity of the collagen lamellar structure.

3. Evolutionary Adaptations in Non-Mammalian Vertebrate Corneas.

While there is no fossil record of vertebrate corneal evolution, we have evaluated the corneal structure of extant species from different vertebrate clades to identify progressive modifications in the collagen organization from fish, amphibians, reptiles, and birds that might help identify key evolutionary adaptations in collagen structure in non-mammalian vertebrate corneas that control corneal shape. From these studies, 3 key structural features were identified. First, corneal stromal structure is built upon an orthogonal/rotational structural paradigm, where stromal collagen exists in layers comprised of parallel fibers that are rotated relative to each other by just less than 90^{o} angles (Figure 5). When viewing the SHG signals taken parallel to the corneal surface or *en face*, the directionality of each layer can be imaged as the plane of focus moves from the anterior lamellae (Figure 5A, 114 µm) to the adjacent layer (Figure 5C, 129 µm). At the interface between the two layers (Figure 5B, 123 µm), collagen fibers in both layers are detected, showing two distinct angles. This angle can be measured using fast fourier transform (FFT) analysis of the image to detect the major orientation of

the structures within the image (Figure 5D). Using this analysis, the Great White Shark cornea showed an angular rotation of 82.6º ± 5.5º. Overall, this angular rotation in fiber directionality between adjacent collagen lamellae appears highly conserved, ranging from 88.2º to 77.7º (Figure 6) in the non-mammalian corneas from the different clades that were studied (Winkler et al., 2015). This finding is in agreement with the orthogonal/rotational collagen paradigm first proposed by Trelstad and Coulombre in 1971 as identified in developing chick corneas (Trelstad and Coulombre, 1971). Later studies by Trelstad also detected angular rotation in other species including turtle, frog and fish (Trelstad, 1982); however, the measured rotation was highly variable using plastic sectioning and silver staining of tissue.

The second major structural feature was that collagen lamellae extended as sheets or ribbons from limbus to limbus in all non-mammalian vertebrate corneas. This is detected by HRMac imaging of the Great White shark cornea, where SHG imaging of vibratome sections show a banded pattern in the stroma (Figure 7). Individual bands represent individual lamella or collagen sheets, for which the strength of SHG signal is dependent on the orientation of the collagen fibrils comprising the lamellae. The 16 *en face* corneal images show the direction of different lamellae as a function of depth from anterior to posterior cornea. Note that lamellae showing collagen fibers oriented in-plane (images 1,3,12 and 14) are associated with very strong SHG signals in the cross-sectional image, while lamellae with collagen fibers oriented out-of-plane (2, 4, 13, and 15) show no signal in the cross-sectional image. Lamellae at different angels produce proportionally stronger or weaker signals. Importantly, the collagen fibers seem to extend complete across the cornea in a single lamella. This is more clearly shown in the HRMac images taken of Falcon cornea and the Atlantic Sharpnose shark (Figure 8) that extend from limbus to limbus and show that a single lamellae oriented in-plane stays at the same depth and extends the entire diameter of the cornea. Overall, this structural organization recapitulates the structural organization of plywood, which is composed of orthogonally arranged wood veneers that are bonded together. One could speculate that this engineered structural design is much stronger to bending stress than a single piece of wood of similar thickness, suggesting a similar functional role for the cornea, perhaps as protection against blunt force trauma.

The third and most important major finding was that there was a progressive increase in the complexity of the collagen lamellae moving from lower (fish) to higher (reptiles and birds) vertebrates as shown in Figure 9. In the Great White Shark, a secondary structure of thin, perpendicular 'sutural' fibers (Figure 9A, small arrow), previously noted by Payrau in 1965, was detected along with very thin branching of lamellae that extended down to adjacent lamellae running in a parallel plane (lower panel, large arrow). Beyond this comparatively rare branching, there were no apparent links between collagen sheets. Bony fish (Figure 9B, Sturgeon), which lacked 'sutural' fibers, also showed rare, thin lamellar branches similar to that seen in cartilaginous fish. The presence of lamellar branching doubled in amphibians (Figure 9C, Bullfrog, lower panel, arrow), increased markedly in number and thickness in reptiles (Figure 9D, Alligator), and was over 10 fold higher in number in birds (Figure 9E, Falcon) compared to fish (Winkler et al., 2015). Lamellar branching and interweaving has been previously associated with increased interlamellar cohesive strength (Maurice, 1988; Smolek 1993; Smolek and McCarey, 1990). As proposed by Smolek and McCarey in 1990, differences in lamellar interweaving modulate the shear strength of the cornea and perhaps control corneal shape. The observed increase in lamellar interconnections detected in higher vertebrates would therefore be consistent with structural adaptations required to control corneal shape to predictably refract light back to the retina.

4. Collagen Structural Organization of the Mammalian Cornea.

Recent NLO SHG studies of lamellar organization in mammalian corneas (Figure 10) have shown that mammals diverged from other vertebrates and that lamellae were organized into ribbons rather than sheets, were seemingly randomly orientated with no discernible orthogonal pattern (Figure 10 A and B), and were no longer constrained to individual planes (Figure 10 C) (Winkler et al., 2015). This finding is in agreement with that of Polack who also noted a non-orthogonal arrangement of collagen fibrils (Polack, 1961). From a mechanical point of view, the random arrangement of collagen fibrils would have the obvious mechanical advantage of facilitating a uniform distribution of strain at each corneal depth resulting in a more stable structure than that achieved by an orthogonally arranged collagen pattern requiring rotation to achieve uniform strain. In

addition, with a random organization the collagen fiber interactions are not limited within a single plane; instead they can traverse through the corneal stroma to interact with lamellae at greater distances. It should also be appreciated that while birds (Coulombre, Coulombre and Mehta, 1962), reptiles (Lee and Szena, 2005) and fish (Mullaney, Coffey and Fenton, 1971) have the bony ossicle at the corneoscleral interface, mammals lack this. Therefore, mammalian corneas need to be self-stabilizing, a feature offered by a random ribbon lamellar organization with branching and anastomosing patterns.

While corneal development in mammals and vertebrates shows distinct developmental similarities, in non-mammalian vertebrates a primary stroma is laid down by the corneal epithelium, which is thought to dictate the alignment of the mature secondary stroma by the presumptive keratocytes, perhaps accounting for the distinct orthogonal arrangement (Conrad, Paulsen and Luer, 1994; Hay and Revel, 1969; Trelstad, 1970; Trelstad and Coulombre, 1971). In mammals, the epithelium does not lay down a primary stroma, and corneal stromal development is thought to involve only the corneal keratocytes (Cintron, Covington and Kublin, 1983). This developmental paradigm might explain the random lamellar organization, although data from x-ray scattering studies support that there is a preferential orientation of collagen fibrils at the superior-inferior and nasal-temporal meridian, which as the authors speculated is directed by mechanical forces (Meek and Boote, 2009).

4.1 Human Corneal Collagen Macrostructure

3D reconstruction of the collagen lamellar organization in the human, as well as other mammalian species, shows a more complex branching pattern than that identified for non-mammalian vertebrates (Morishige et al., 2006; Winkler et al., 2011). As shown in Figure 11, segmentation of lamellae in the anterior corneal stroma shows a markedly higher degree of branching of segmented lamellae from the midstroma, whereas lamellae in the posterior part of the stroma exhibited almost no branching at all. 3D reconstructions also identified other distinct collagen fiber structures the first of which were long, prominent lamellae originating at or near the limbus that extended for several millimeters across large portions of the cornea (Figure 12). These fibers did not follow

the corneal curvature from limbus to limbus; instead they traversed upwards for 100s µm before terminating at or near Bowman's layer. Interestingly, evaluation of the apparent complexity of these lamellae suggest that as these lamellae traversed toward the anterior stroma, the number and complexity of lamellar branching increased, such that in the anterior stroma the individual lamellae could no longer be identified as they joined with the highly branched anterior stromal lamellar meshwork.

A second type of structure identified was the 'Bow spring'-like lamellae that originated from the highly intertwined lamellae directly beneath Bowman's layer and arced upwards, fusing with the Bowman's layer before arcing back down (Figure 13; blue = Bow spring fibers, yellow = Bowman's layer). 'Bow spring' lamellae were therefore characterized by a near-parabolic shape, the apex of which appeared fused with Bowman's layer (Winkler et al., 2011). In part these 'Bow spring' lamellae represent a dual 'suture' lamellae identified earlier by Morishige et al, as angled collagen fibers (Morishige, 2007). These 'Bow spring' or angled collagen fibers which insert into Bowman's layer may have important mechanical functions and play a role in defining corneal shape and curvature in that SHG imaging of corneas obtained from patients with keratoconus, an eye condition in which the cornea thins and acquires a cone-like shape, show a distinct decrease in this population of lamellae. Likewise, SHG imaging shows much less lamellar interweaving in keratoconus corneas compared to normal corneas. The decrease in the number of angled collagen fibers becomes more prominent in the deeper stromal layers while lamellar interweaving is much less apparent. 3D analysis of the forward SHG signal from *en face* images further supported that in keratoconus corneas there is a dramatic decrease in angled or branching fibers and when present only penetrated a short distance into the anterior stroma.

Overall, these findings support the notion that the arrangement of branching and angled collagen fibers that provides mechanical strength and rigidity to the anterior stroma may also determine corneal shape and curvature, a concept first proposed by Sir William Bowman (Bowman, 1849). The loss of this collagen lamellar complexity leading to decreasing interlamellar connectivity would subsequently promote lamellar slippage, a long-postulated alternative pathological mechanism of keratoconus (Meek et al., 2005; Hayes et al., 2007, 2012; Smolek and Klyce 2000). Indeed, recent x-ray studies of

keratoconus corneas propose lamellar slippage as a potential mechanism of keratoconus progression (Meek et al., 2005; Hayes et al., 2007).

4.2 Collagen lamellar organization as a function of corneal depth

As mentioned earlier, there are significant differences in the lamellar architecture and branching patterns as a function of corneal stromal depth in the human cornea (Morishige et al., 2006, 2007; Quantock et al., 2007). Lamellae at the anterior stroma exhibit extensive branching and anastomosing patterns and the majority of angled fibers run transverse to the corneal surface (Figure 14A). Deeper in the corneal stroma, lamellae exhibit little branching, running parallel to the surface of the cornea. X-ray studies of albeit swollen human corneas also suggested structural lamellae heterogeneity in different depths of the cornea (Quantock et al., 2007). It is well acknowledged that the anterior stroma is more resistant to corneal swelling, which has also been related to the collagen lamellar interweaving suggesting increased mechanical stiffening (Muller et al., 2001). These findings further suggest that lamellar architecture controls the material properties of the corneal stroma (Bron, 2001; Muller et al., 2001).

More recently studies have extended this hypothesis and shown that lamellae are not only more interwoven but are also more intertwined, with fibers splitting and joining other fibers in many adjacent lamellae, which would offer additional rigidity and stiffness to the tissue (Morishige et al., 2006, 2007; Winkler et al., 2010). In quantitative studies measuring the density of collagen fiber branching and anastomosis versus corneal stromal depth and location (Winkler et al., 2011, 2013), lamellar complexity in both the central and peripheral cornea was shown to be highest in the anterior stroma compared to the posterior stroma. The highest lamellar branching density (37 branches/mm) was noted at the anterior part of the stroma, immediately below Bowman's layer. The detected amount of lamellar branching appeared to decrease as you move deeper in the stroma, representing an average branching point density of 3 branches/mm, right above the corneal endothelium (Figure 14B).

Lamellar angles most likely have clear implications in tissue stiffness as they could exert vectorial tensor effects which can influence tissue response to compressive or shear

strain. Calculation of fiber angle is an alternative method for measuring lamellar branching and measurement of fiber angles shows greater angular distribution in the anterior stroma than the posterior, while there is no difference in the radial distribution by region or quadrant of the cornea, suggesting that there are no major differences in the lamellar structure from centre to periphery (Winkler et al., 2011, 2013).

These structural differences between anterior posterior and central peripheral are also consistent with regional measurements of mechanical stiffness. In studies evaluating the indentation modulus of corneal flaps taken from the anterior, mid and posterior human corneal stroma (Winkler et al., 2011), the anterior flaps showed a 2-3 fold higher resistance to indentation than the mid and posterior flaps, recapitulating the differences measured in lamellar branching (Figure 15). These mechanical findings have been verified by others using a range of different techniques, including acoustic radiation force elastic microscopy (Mikula et al., 2014; Mikula, Jester and Juhasz, 2016), atomic force microscopy (Last et al., 2009, 2012; Thomasy et al., 2014), and Brillouin microscopy (Randleman, Su and Scarcelli, 2017; Scarcelli and Yun, 2012; Scarcelli et al., 2013; Scarcelli, Pineda and Yun, 2012) as well as others (Dias and Ziebarth, 2013; Last et al., 2012; Petsche and Pinsky, 2013) emphasizing the effects of collagen architectural organization on mechanical tissue stiffness and shape. In particular, these studies propose that collagen lamellar branching and anastomosing along with angled lamellae greatly influence tissue mechanics and control corneal shape in the mammalian cornea. From all these studies it appears apparent that the mammalian cornea, and particularly the human cornea, is a self-supporting structure, that is composed of an interconnected network of collagen fibers extending anterior-posterior, central-peripheral, providing a structurally defined refractive shape (Figure 16).

5. Future Directions

Over the years, state of the art technologies, from electron microscopy to x-ray scattering and HRMac of SHG signals, have been extensively used by various researchers to characterize the 3D collagen architecture of the corneal stroma and relate its structure to tissue form and function. In this review we have identified a progression of structural changes in the cornea from lower (fish) to higher (birds and mammals) vertebrates that

suggest architectural adaptations necessary for controlling corneal shape to accommodate terrestrial vision. These modifications have taken the form of replacing the plywood-like structural organization of rotating collagen sheets that provide a covering function for the cornea, with a complex collagen lamellar organization with branching and anastomoses that presumably transforms a flat cover into a convex refractive lens (Figure 17). Importantly, these evolutionary changes that are greatest in birds and humans that also have the highest visual acuity.

Aside from structural changes which appear to be a milestone during evolution of the cornea, it is reasonable to speculate that changes at a molecular or genetic level, such as changes in the genes controlling proteoglycans and other extracellular matrix proteins may also play an evolutionary role and should be further investigated in the future. In addition, the mechanisms (cellular, molecular and mechanical) that control branching and anastomosing of collagen lamellae and the overall organization of the corneal stroma remain unknown, and in the end " the weaving of the body fabric from the warp and woof of the matrix has yet to told (Trelstad and Birk, 1984)". While findings support that collagen organization potentially affects the biomechanical properties of the cornea; the relationship, if any, with respect to corneal topography and the convex anterior surface of the cornea remains unknown. Future investigations mapping collagen architecture in vivo to the in vivo topographic data may perhaps uncover the exact mechanism by which collagen organization determines corneal curvature and shape. Filling these knowledge gaps will lead to a more comprehensive understanding of the hierarchical structure of the corneal extracellular matrix and in the future, will potentially aid the development of more effective refractive corrective procedures, and point towards the development of bioengineered tissues that more realistically recapitulate normal tissue structures.

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Figure Legends

Figure 1: Human collagen lamellae imaged using transmission electron microscopy (A) and backscatter SHG (B). (A) Conventional transmission electron micrograph shows that collagen fibrils are organized into orthogonally arranged lamelle. Scale: 500 nm (B) An SHG signal is only detected when the collagen lamellae are running "in plane" and hence longitudinally section collagen lamellae show prominent SHG signal, while orthogonally arranged lamellae above and below show a weak SHG signal when viewed in cross section. Scale: 50 µm

Figure 2: Tissue embedding in low melting point agarose and vibratome sectioning. (A) Vibratome sectioning of a human cornea embedded in low melting point agarose. (B) Agarose block containing human cornea sample glued to a metal block. (C) Vibratome section of human cornea extending from limbus to limbus. (Adapted from Jester JV, Winkler M, Jester BE, Nien C, Chai D, Brown DJ. 2010. Evaluating corneal collagen organization using high resolution non linear optical (NLO) microscopy. Eye Contact Lens. 36: 260-264)

Figure 3: Image concatenation into a 2 x 2 mosaic. Individual single-plane images show forward scatter (cyan) and backscatter (purple) SHG signal (human cornea). The image concatenation process merges image stacks sequentially based on a per-plane basis with the use of the microscope stage coordinates. Minor misalignments due to stage and sample drift are corrected using an image registration algorithm. (Adapted from Winkler M, Jester BE, Nien-Shy C, Chai D,Brown DJ, Jester JV. 2010. High resolution macroscopy (HRMac) of the eye using non-linear optical imaging. Proc. SPIE 7589 (758906), 758901-758907).

Figure 4: HRMac image of a human corneal cross-section along the vertical meridian and 3D reconstructions of collagen fibers. (A) Single-plane HRMac image of full diameter corneal cross-section made up of 80 Mega Pixel image planes (backscatter SHG). Scale = 1 mm. (B) 3D reconstructed selected collagen sutural

lamellae (purple) beneath Bowman's layer (green) overlaying the HRMac image (backscatter SHG). (C) Extracted segmented lamellae visualized in different orientations. (Adapted from Winkler M, Jester BE, Nien-Shy C, Chai D, Brown DJ, Jester JV. 2010. High resolution macroscopy (HRMac) of the eye using non-linear optical imaging. Proceeding of SPIE, Vol 7589: 758906.).

Figure 5: *En face* forward SHG imaging of the great white shark cornea. Single-plane SHG image taken at a stromal depth of (A) 114 µm, (B) 123 µm and (C) 129 µm from the corneal epithelial layer. (D) The FFT analysis of the collagen orientation at the interface between collagen sheets, showing an orthogonal organization. Scale = 50 µm. (Adapted from Winkler M, Shoa G, Tran ST, Xie Y, Thomasy S, Raghunathan VK, Murphy C, Brown DJ, Jester JV. 2015. A comparative study of vertebrate corneal structure: The evolution of refractive lens. Invest Ophthalmol Vis Sci. 56: 2764-2772)

Figure 6: 3D FFT reconstructions of SHG image stacks of (A) great white shark, (B) salmon, (C) bullfrog, (C) alligator and (E) falcon. Plane-by-plane FFTs were computed and 3D reconstructed for each species to illustrate collagen fiber directionality within each layer of the corneal stroma. (F) The angular displacement as a function of stromal depth for each species showing that in nonmammalian corneas there is over 100° to 200° collagen rotation. The collagen rotation stops in the posterior layers for only the reptiles and birds (arrows). (Adapted from Winkler M, Shoa G, Tran ST, Xie Y, Thomasy S, Raghunathan VK, Murphy C, Brown DJ, Jester JV. 2015. A comparative study of vertebrate corneal structure: The evolution of refractive lens. Invest Ophthalmol Vis Sci. 56: 2764-2772)

Figure 7: HRMac image of great white shark corneal cross-section. Imaging of vibratome cross-sections show a banded SHG signal. The corresponding directionality of the collagen lamellae at each band are shown (1-16, arrows, forward SHG). Scale = 1 mm. (Adapted from Winkler M, Shoa G, Tran ST, Xie Y, Thomasy S, Raghunathan VK, Murphy C, Brown DJ, Jester JV. 2015. A comparative study of vertebrate corneal structure: The evolution of refractive lens. Invest Ophthalmol Vis Sci. 56: 2764-2772)

Figure 8: HRMac imaging of the (A) falcon and (B) the Atlantic sharpnose shark. HRMac reconstructions of vibratome corneal sections of the falcon and the Atlantic sharpnose shark show that collagen fibers in a lamellar plane extend across the entire cornea from limbus to limbus (backscatter SHG). Scale = 1mm. (Adapted from Winkler M, Shoa G, Tran ST, Xie Y, Thomasy S, Raghunathan VK, Murphy C, Brown DJ, Jester JV. 2015. A comparative study of vertebrate corneal structure: The evolution of refractive lens. Invest Ophthalmol Vis Sci. 56: 2764-2772)

Figure 9: Cross-sectional HRMac images of corneas from distinct vertebrate species. Full-thickness (top panel) and high-resolution (lower panel) HRMac images of backscatter SHG of the great white shark cornea, the sturgeon cornea, the bullfrog cornea, the alligator cornea and the falcon cornea. The shark cornea shows perpendicular 'sutural' fibers (small arrows), along with thin branching (large arrows). The sturgeon cornea lacks 'sutural' fibers and rarely shows lamellar branching. Lamellar branching is more evident in the bullfrog cornea and even more prominent in the alligator cornea. The bird cornea shows the highest amount of lamellar branching and anastomosis amongst all nonmammalian corneas. Top panel scale = 200 µm, Lower panel scale = 50 µm. (Adapted from Winkler M, Shoa G, Tran ST, Xie Y, Thomasy S, Raghunathan VK, Murphy C, Brown DJ, Jester JV. 2015. A comparative study of vertebrate corneal structure: The evolution of refractive lens. Invest Ophthalmol Vis Sci. 56: 2764-2772)

Figure 10: SHG imaging of mammalian corneas. (A) *En face* forward SHG image of the rabbit cornea and (B) the respective FFT analysis at the interface, showing collagen lamellae to be arranged into ribbons, having a random organization. Scale = 50 µm. (C) Cross-sectional HRMac image of the human cornea showing a dynamic fiber bundle network, with fibers oriented in various directions within and in other layers. Note that branching and anastomosis is not restricted to the nearest neighbors (backscatter SHG). (Adapted from Winkler M, Shoa G, Tran ST, Xie Y, Thomasy S, Raghunathan VK, Murphy C, Brown DJ, Jester JV. 2015. A comparative study of vertebrate corneal structure: The evolution of refractive lens. Invest Ophthalmol Vis Sci. 56: 2764-2772)

Figure 11: 3D surface reconstructions of collagen fibers at different corneal stromal depths. (A) Single-section HRMac plane showing representative segmented collagen

fibers from the anterior (purple) to the posterior central (blue) human cornea (backscatter SHG).. (B) The segmented collagen fibers overlaying the HRMac image plane at decreased opacity. Lamellar branching and anastomosis reduces from anterior to posterior human cornea (backscatter SHG).. (Adapted from Quantock AJ, Winkler M, Parfitt GJ, Young RD, Brown DJ, Boote C, Jester JV. 2015. From nano to macro: studying the hierarchical structure of the corneal extracellular matrix. Exp Eye Res. 133: 81-99).

Figure 12: 3D reconstruction of collagen fibers originating at or near the limbus. 3D reconstructed collagen fibers (green) that originate at or near the limbus extend across large portions of the human cornea (backscatter SHG).

Figure 13: 3D reconstruction of human 'Bow spring' collagen fibers. 'Bow spring' collagen fibers (blue) originate from the highly intertwined lamellae (teal) below Bowman's layer (yellow). (Adapted from Winkler M, Chai D, Kriling S, Nien CJ, Brown DJ, Jester B, Juhasz T, Jester JV. 2011. Nonlinear optical microscopic assessment of 3-D corneal collagen organization and axial biomechanics. Invest Opthalmol Vis Sci. 52: 8818-8827).

Figure 14: Branching point densities as a function of human corneal stromal depth. (A) Fifteen segments of equal length were created along perpendicular lines to identify lamellae that were then followed and the distance to the next branching point measured 3-dimensionally (backscatter SHG). (B) Collagen fiber interconnectivity, expressed as a function of branching point density against corneal stromal depth. Branching point density averaged over five eyes as a function of stromal depth. The solid line shows an exponential curve fit with $R^2 > 0.98$. (Adapted from Winkler M, Chai D, Kriling S, Nien CJ, Brown DJ, Jester B, Juhasz T, Jester JV. 2011. Nonlinear optical microscopic assessment of 3-D corneal collagen organization and axial biomechanics. Invest Opthalmol Vis Sci. 52: 8818-8827).

Figure 15: Regional measurements of mechanical stiffness and the indentation modulus. A) Elastic modulus averaged over seven human corneas grouped by flap position (Anterior, mid, posterior). NS indicates no statistically significant difference between values ($p = 0.6$). (B) Averaged branching point density over five HRMac-imaged

human corneas versus corneal stromal depth (anterior, middle, posterior). NS indicates no statistically significant difference between values (p = 0.05). Corneal flaps were prepared using a surgical femtosecond laser system (Intralase; Abbot Medical Optics Inc., Abbot Park, IL) while still under pressure. (Adapted from Winkler M, Chai D, Kriling S, Nien CJ, Brown DJ, Jester B, Juhasz T, Jester JV. 2011. Nonlinear optical microscopic assessment of 3-D corneal collagen organization and axial biomechanics. Invest Opthalmol Vis Sci. 52: 8818-8827).

Figure 16: The human corneal stroma is an interconnected, self-supporting structure. 3D reconstruction of a human corneal cross-section showing collagen fibers (orange) extending from the anterior-posterior and central-peripheral cornea (green), forming a structurally transparent tissue.

Figure 17: Emergence of a refractive lens throughout the animal kingdom. Evolutionary structural changes in the collagen organization of the corneal stroma are associated with the evolution of the cornea from a cover to a refractive lens. Nonmammalian corneas display a common orthogonal/rotated collagen structural organization that shows increased lamellar branching in higher vertebrates. Mammalian corneas displayed a random collagen structural organization with no orthogonal, chiral pattern and with significant collagen branching.

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