



# Refractive index degeneration in older lenses: A potential functional correlate to structural changes that underlie cataract formation



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## ABSTRACT

A major structure/function relationship in the eye lens is that between the constituent proteins, the crystallins and the optical property of refractive index. Structural breakdown that leads to cataract has been investigated in a number of studies; the concomitant changes in the optics, namely increases in light attenuation have also been well documented. Specific changes in the refractive index gradient that cause such attenuation, however, are not well studied because previous methods of measuring refractive index require transparent samples. The X-ray Talbot interferometric method using synchrotron radiation allows for measurement of fine changes in refractive index through lenses with opacities. The findings of this study on older human lenses show disruptions to the refractive index gradient and in the refractive index contours. These disruptions are linked to location in the lens and occur in polar regions, along or close to the equatorial plane or in lamellar-like formations. The disruptions that are seen in the polar regions manifest branching formations that alter with progression through the lens with some similarity to lens sutures. This study shows how the refractive index gradient, which is needed to maintain image quality of the eye, may be disturbed and that this can occur in a number of distinct ways. These findings offer insight into functional changes to a major optical parameter in older lenses. Further studies are needed to elicit how these may be related to structural degenerations reported in the literature.

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## 1. Introduction

The power of a lens to refract light is the prime measure of its functional capacity. In the case of the biological lens, its refractive power, which depends on the curvature and refractive index is linked to the distribution and concentration of its constituent proteins (Pierscionek and Augusteyn, 1991, 1992; Keenan et al., 2008, 2009; Grey and Schey, 2008; Slingsby et al., 1997 Slingsby, 1985; Pierscionek and Regini, 2012). The functional capacity of the lens to refract is contingent on relatively unimpeded passage of light through its medium. When cataract develops and disrupts the transmission of light through the lens, the structure/function relationship between the proteins and the optical properties is altered. Histological and microscopic studies that have looked at fine structure in lenses with cataract, or in older lenses that show

changes similar to those found in cataractous lenses, indicate the major structural degradations that underlie the process of opacification (Costello et al., 1992, 2008; Gilliland et al., 2004; Al-Ghoul and Costello, 1993, 1996; Al-Ghoul et al., 1996; Metlapally et al., 2008; Costello et al., 2012; Brown et al., 1989, 1993; Vrensen and Willekens, 1990; Bassnett et al., 2011). Conclusions have been drawn about how structure maintains transparency and hence what structural degradations may affect loss of function (Bassnett et al., 2011) but these structural studies have not been complemented with investigations of functional, optical parameters to the same level of detail.

Whilst it is known that the two major attenuation factors that manifest clinically as cataract, are scatter and absorption of light, there is a paucity of studies on the optics of intact lenses and the changes in the refractive index that may indicate early stages of opacification. This is because methods of measuring refractive index require or assume that light will be refracted and not scattered or absorbed (Pierscionek and Regini, 2012). One study that used

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fibre optic sensing to measure refractive index from the amount of reflected light in very localised regions of the lens, found significant attenuation in the reflections from central regions of older lenses and concluded that these most likely indicated optical changes caused by structural degradation (Pierscionek, 1997).

To measure with accuracy how refractive index changes in older and cataractous lenses requires a method that can detect localised refractive index fluctuations without affecting measurements in regions of the lens that can still transmit light. The recent development of an interferometric method using a synchrotron X-ray source has made it possible to measure refractive index directly and accurately in any given plane of whole lenses within the eyeball (Hoshino et al., 2011). This method can detect fluctuations in refractive index that show structural degradation and differentiate these from normal fluctuations in the refractive index gradient (Hoshino et al., 2011; Bahrami et al., 2014) because it does not require the sort of assumptions necessary in other methods that have been used to determine or estimate refractive index in intact lenses (reviewed in Pierscionek and Regini, 2012).

This study on refractive index variations in older donor lenses has found functional characteristics that show forms of deviation from the refractive index gradient beyond the physiological fluctuations found in healthy younger samples (Bahrami et al., 2014). The deviations indicate abnormalities in the refractive index gradient that may be relevant to structural degradations and provide the basis for defining and understanding early cataractous changes in the optical parameters of the lens.

## 2. Methods

Older human eyes (15 samples aged from 72 to 88), from which corneal discs were removed for transplantation, were obtained from the Bristol Eye Bank (UK) and transported in dry ice to the SPring-8 synchrotron radiation facility in Japan where they were placed at  $-20\text{ }^{\circ}\text{C}$ . Samples were moved in pairs from  $-20\text{ }^{\circ}\text{C}$  to  $+4\text{ }^{\circ}\text{C}$  at 12–13 h before measurement and placed at room temperature around 3 h before measurement. The same procedure was applied to all samples in order to maintain similar pre-experimental conditions. Thawed lenses were removed from eye-balls and set in an agarose gel that was physiologically balanced, within a specially designed sample holder. Samples were set in pairs, one above the other. Refractive index variations was measured using the X-ray Talbot grating interferometer (Momose, 2005; Momose et al., 2003) as described previously (Hoshino et al. (2011); Bahrami et al., 2014). The interferometer uses a 25 keV X-ray beam at the bending magnet beamline BL20B2. X-rays pass through a Si(111) double crystal monochromator and two transmission gratings. The first was a tantalum phase grating (G1) with pattern thicknesses  $2.1\text{ }\mu\text{m}$  and the second a gold absorption grating (G2) (pattern thickness of  $16.6\text{ }\mu\text{m}$ ). Both gratings have a grating pitch of  $10\text{ }\mu\text{m}$  and a pattern size area of  $25\text{ mm}$ . Moire fringes are detected by the beam monitor and a scientific CMOS detector (ORCA Flash 4.0. Hamamatsu Photonics). A Piezo stage was used to shift G2 for phase retrieval and a 5-step 'on-the-fly' fringe-scan method was used. Phase shifts were calibrated against solutions of known density and experimental results compared to phase shifts per pixel which were converted to refractive index as described in Hoshino et al. (2011). The measurement on each sample took 50 min providing 900 scans for each lens. Repeat measurements were conducted on two lenses. The error in the measurement of refractive index was  $\pm 0.0005$ .

Refractive index values in three-dimensional spatial coordinates were processed using Mathematica computational software v9 to plot iso-indicial contours for increments of 0.002 in refractive index. The study was approved by the National Health Service (NHS)

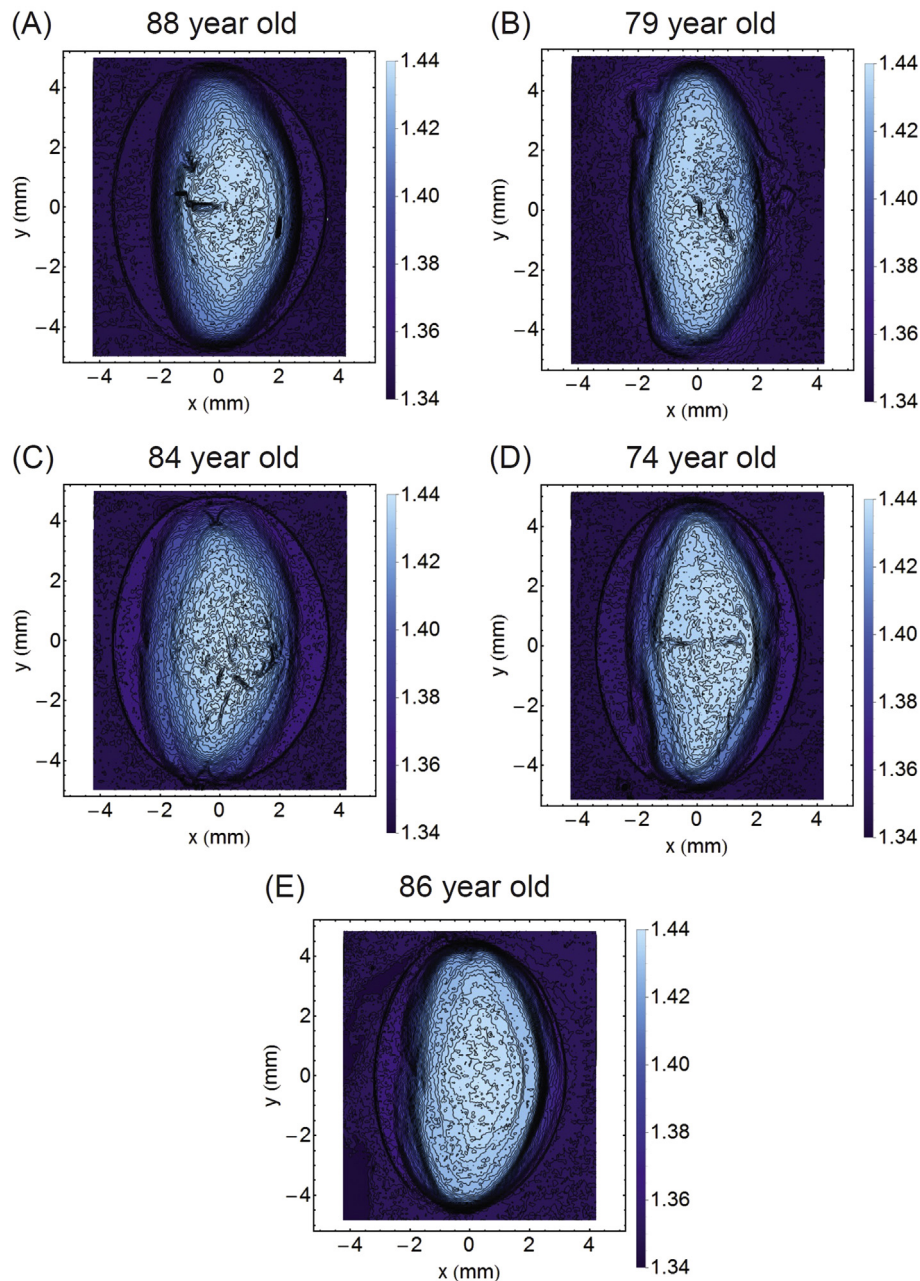
ethics committee (Oxford, UK).

## 3. Results

Fig. 1A to E shows refractive index contours in sagittal planes of five lenses. Changes in the colour density represent changes in refractive index with each adjacent contour indicating an incremental step changes of 0.002 in refractive index. In four of these figures, notably Fig. 1A to D, the refractive index contours are distorted or show localised areas of relatively large density fluctuations that represent disturbances in the refractive index profiles. These disturbances are categorised into four types: those that occur in the region of the optic axis (three lenses with representative shown in Fig. 1A), those that are located close to or within the equatorial plane (five lenses with representative shown in Fig. 1B), those that follow directions that are oblique to the optic axis and the equatorial plane and are close to the equatorial region (four lenses with representative shown in Fig. 1C) and those that appear as denser layers: ie disturbances that run along contours that approximate the outer shape of the lens (three lenses with representative shown in Fig. 1D). In four lenses more than one type of disturbance was found. This is seen in Fig. 1D that shows evidence of disturbances which appear to follow lamellae as well as slight density changes in the polar region. The lamellar-type patterns are also seen with density fluctuations in the equatorial plane or in planes oblique to the optic axis. There is no evidence, within those lenses that have disturbances in the polar regions, of any fluctuations that run parallel or within the equatorial plane. Fig. 1E shows the refractive index contours for an 86 year old lens with no evidence of significant disturbances to the index contours indicating that these changes are not found in all older lenses and are therefore most likely to be a manifestation of changes that may be indicative of early opacification and underlying cataractous processes rather than ageing.

The refractive index profiles in the sagittal plane for the lenses given in Fig. 1 are shown in Fig. 2. The fluctuations in the nucleus are seen in Fig. 2A–D which correspond to samples where perturbations in the refractive index contours are seen. The changes are most dramatic for the profile in Fig. 2A which shows a sharp cleft in the refractive index corresponding to the density disturbances seen in Fig. 1A. The refractive index dips to around 1.412 with maxima of 1.426 and 1.435 seen on either side. In Fig. 2B and C where the refractive index disturbances are, respectively, parallel to the equatorial plane and in orthogonal directions, the refractive index profiles have sharper drops and the profiles are distorted. Fig. 2D, which corresponds to the refractive index profile with disturbances that run along contours, has no fluctuations in refractive index in the sagittal plane that are beyond those seen in younger lenses (Bahrami et al., 2014). Instead, consistent with lamellar density changes (Fig. 1D), there is a sharp definition between the almost flat portion of the refractive index profile and the cortical regions that show a steep gradient with fluctuations at the intersections. A slight distortion of the profile is also evident with a steeper cortical gradient in the posterior than in the anterior part of the lens. The refractive index profile in Fig. 2E is relatively smooth and symmetrical, akin to that seen in younger lenses (Bahrami et al., 2014) with some small fluctuations in the central, nuclear part of the profile.

The corresponding refractive index contours in the equatorial plane for the same set of lenses are given in Fig. 3. Fig. 3A, which corresponds to disturbances in refractive index in the polar regions, has very few fluctuations but a slight elevation in refractive index at around 2 mm on either side of the centre of the profile (marked with arrows) and a sharp dip in the centre of the profile (marked with an arrow). Where disturbances occur within the equatorial

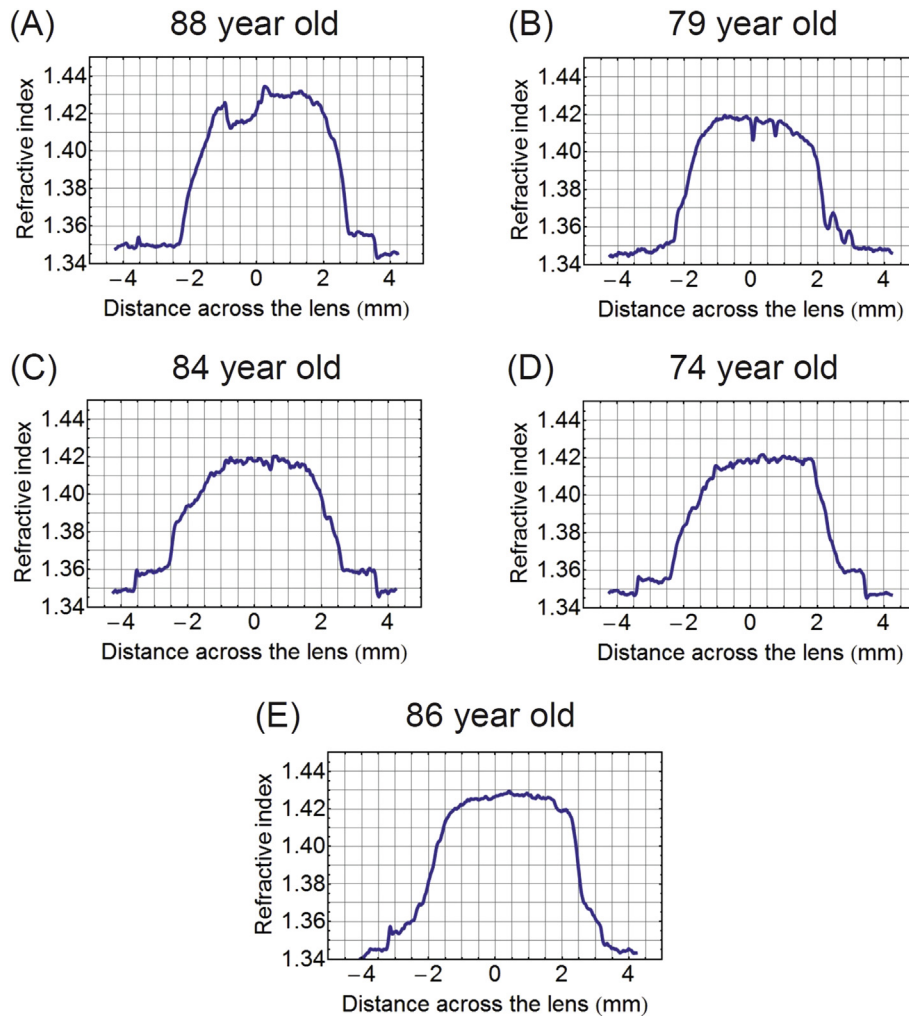


**Fig. 1.** Refractive index contours in the sagittal planes of five human lenses aged A) 88 years; B) 79 years; C) 84 years; D) 74 years and E) 86 years of age showing different patterns of disturbance to the contours. Changes are seen in polar regions in A) and to a lesser extent D); along the equatorial region in B) in oblique spokes in C) and in lamellar formation in D) and to a lesser extent in A) and C). The 86 year old lens in E) represents the healthy lens. Refractive index contours are shown in increments of 0.002 with changes in colour representing the changes in refractive index on the scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

plane, the fluctuations on the refractive index profile are more evident (Fig. 3B and C). In Fig. 3B, there is a sharp dip in the centre of the profile and there is a mismatch in the refractive index values in the plateau region on either side of this dip: the refractive index is slightly higher on the anterior side compared to the posterior side. Fig. 3C has a number of fluctuations in the central part of the profile that do not show any regularity or symmetry on either side of the centre. The equatorial profile in Fig. 3D has a similar form to its sagittal counterpart (Fig. 3D) with relatively sharp definition between the flat portion of the profile and very steep gradient sections compared to all the other samples. The profile in Fig. 3E is regular and resembling that of a healthy lens (Bahrami et al., 2014).

The irregularities in refractive index in the polar regions in the

88 year old lens (Fig. 1A) appear to be branching in the anterior section of the lens with two branches running above and below the optic axis region and one branching into the lens. Progression through this lens from anterior to posterior poles, in incremental steps of 1.98 mm, shows patterns that are similar to sutures (Fig. 4). The characteristic Y-shape suture (Fig. 4A) becomes denser with progression into the lens (Fig. 4B), more distorted (Fig. 4C) and branching (Fig. 4D) and then appearing as an inverted Y-shape (Fig. 4E and F) relative to the suture seen in Fig. 4A. The sutural patterns are more pronounced in an 85 year old lens with denser opacities around the optic axis and polar regions (Fig. 5D–F). In this lens, where each incremental step is 0.684 mm apart, the Y-shaped pattern is evident in the anterior parts of the lens (Fig. 5A) and into



**Fig. 2.** Refractive index profiles in the sagittal planes of five human lenses aged A) 88 years; B) 79 years; C) 84 years; D) 74 years and E) 86 years of age plotted against distance across the lens from the anterior (negative numbers) to the posterior (positive numbers) poles.

the nuclear region (Fig. 5B and C) becoming highly branched (Fig. 5D) and much denser toward the posterior pole (Fig. 5E and F). The sagittal refractive index contours and the refractive index profile for this lens are shown in Fig. 5G. There are high density fluctuations in the posterior polar region and along the optic axis that are consistent with the patterns seen in Fig. 5A–F.

Fig. 6A and C shows the refractive index contours from initial scans for 86 and 88 year old lenses, respectively, alongside results from the same lenses, acquired from repeat scans (Fig. 6B and D). The repeat scans are almost identical to the initial scans.

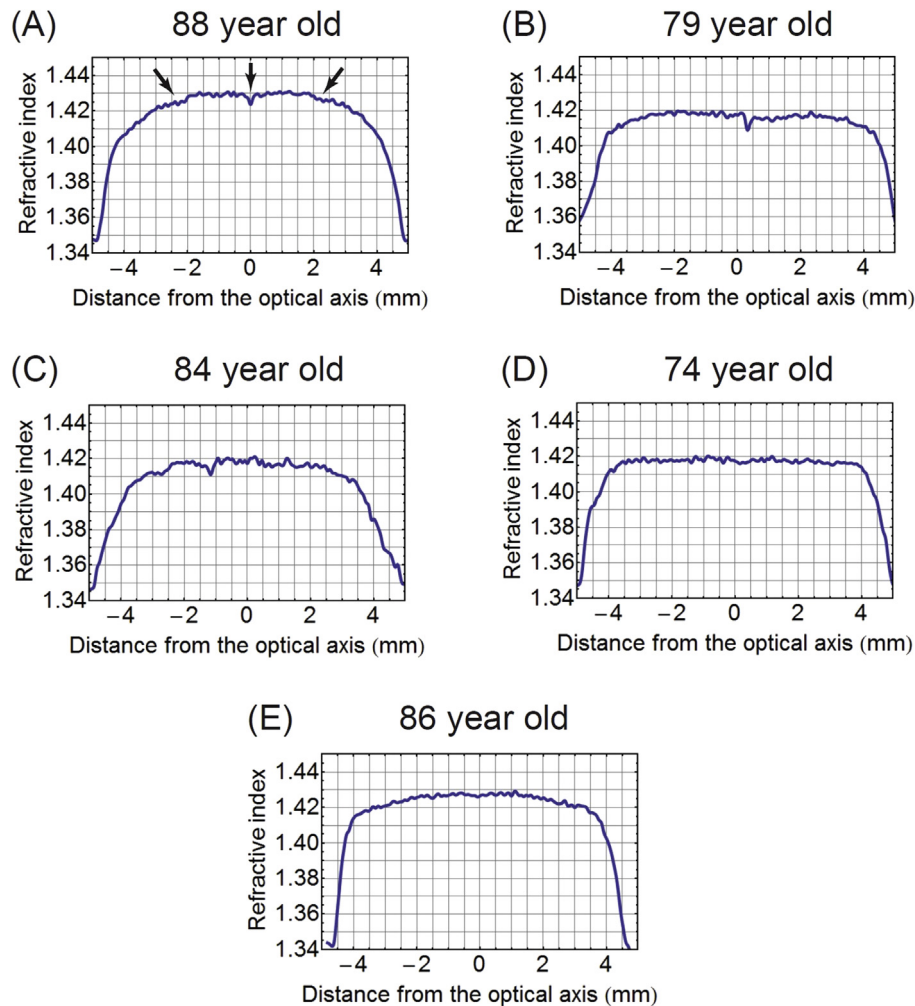
#### 4. Discussion

The refractive index of the eye lens follows a gradient which in the human lens occurs largely in the cortex with little variation in the central nuclear regions (Pierscionek and Chan, 1989; Pierscionek and Regini, 2012). Recent research has discovered that the refractive index does not vary smoothly but shows kinks and discontinuities (Hoshino et al., 2011; Bahrami et al., 2014) that are beyond the error range. When these discontinuities were incorporated into an optical model that mimicked the way in which light is reflected and backscattered from the living lens viewed through a slit-lamp biomicroscope, the simulations produced the ring-like characteristics, termed zones of discontinuity that are

seen in the living lens (Bahrami et al., 2014). This suggests that the refractive index fluctuations are linked to the zones of discontinuity but whether these irregularities in the refractive index profile have a specific optical function or whether they arise from the growth mode of the lens and irregular rates of tissue accrual, as is seen in growth rings of a tree, is not known (Pierscionek and Regini, 2012). It has been suggested from a previous study, in which fluctuations in the refractive index gradients of fish lenses were found, that these define zones of different focal lengths and may have a function in controlling chromatic aberrations (Kroger et al., 1999). The mechanism of how this may occur has not been shown and whether or not the refractive index fluctuations serve to increase image quality, these deviations and the presence of zones of discontinuity, are not detrimental to vision. When density variations become more irregular, manifesting as scattering centres or spokes in the living lens, the transmission of light is impeded. This arises because protein and/or lipid aggregates are of such a scale that the refractive index fluctuations cause light to scatter (Tang et al., 2003; Gilliland et al., 2004; Costello et al., 1992).

The findings from this study show that relatively large disturbances that deviate from the normal variations in protein density that form the refractive index gradient, appear to follow certain patterns that are based on location and to a lesser extent orientation of these irregularities. The fluctuations in density that run





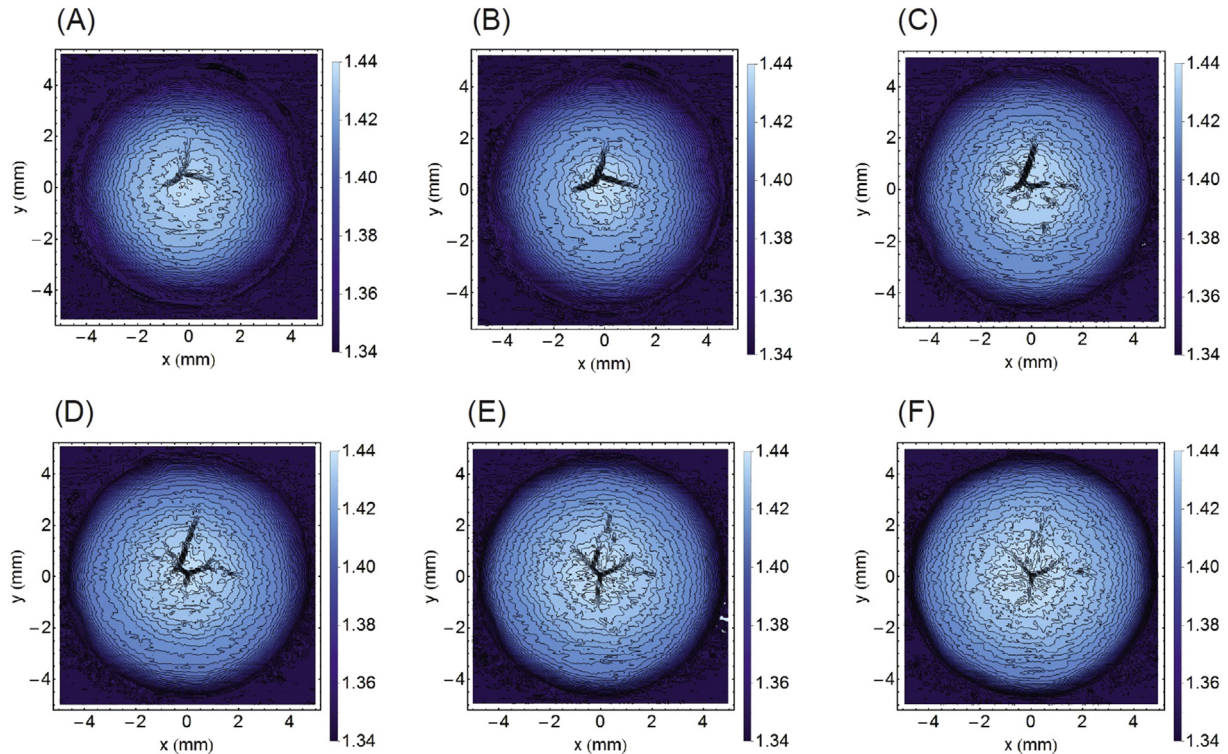
**Fig. 3.** Refractive index profiles in the equatorial planes of five human lenses aged A) 88 years; B) 79 years; C) 84 years; D) 74 years and E) 86 years of age plotted against distance from the optical axis. Arrows in a) indicate abrupt changes to which reference is made in the text. Some of the samples (notably in B) 79 years and C) 84 years) were larger than the field of measurement and refractive index values at the extreme edges could not be measured.

approximately parallel or in oblique directions relative to the equator of the lens (Fig. 1B and C) suggest a type of spoke opacity. These types of opacities generally appear in the cortical layers of the lens when viewed in the living eye (Brown et al., 1989; Brown et al., 1993; Pau, 2005). Lenses from donor eyes with cuneiform cortical cataracts viewed in dark field illumination also showed spoke opacities but these were outside the pupillary region (Michael et al., 2008) and therefore may not be directly correlated to what is seen in Fig. 1B and C. Histological studies of nuclear cataracts have found subtle disruptions in cellular architecture in the form of fluctuations in protein density between cell layers and changes between membranes and extracellular space (Costello et al., 1992). Such changes at the cellular level are of a much smaller scale than the refractive index fluctuations in this study and hence a correlation between the structural and functional changes cannot be made without further investigations.

Cortical opacities seen in the equatorial aspects of older donor lenses (aged between 55 and 90 years) have been reported to be of two distinct types: circular, sharp and clearly defined shades following the outer lens shape and less well defined radial opacities (Michael et al., 2008). These two types of opacities are frequently seen close together. In previous microscopic studies, Vrensen and Willekens (1990) identified two types of early cortical opacities in

the ageing lens: radial and circular shades, the former located in the deep cortex and affecting small groups of fibres, the latter involving a larger number of lens fibre cells and running perpendicular to the fibres in the equatorial plane. In severe cases circular shades extended towards either the anterior or posterior poles (Vrensen and Willekens, 1990).

The opacities identified by Michael et al. (2008), that are largely seen in the equatorial region of the cortico-nuclear boundary, have been linked with mechanical stresses caused by the effort of shape change during the act of accommodation (Fisher, 1970). The assumed differences in mechanical properties between the nuclear and cortical regions are hypothesised as producing shear stresses when the lens shape changes (Michael et al., 2008). Finite element modelling of accommodation (Belaidi and Pierscionek, 2007) based on experimental findings in which human lenses were stretched in a manner simulating the action of accommodation in the eye (Pierscionek, 1995) showed that stresses are indeed greatest in the equatorial region around the cortico-nuclear boundary. It is possible that decades of accommodative effort, prior to the lens losing capacity to alter its shape, render these areas more vulnerable to opacification. It may also be that older lenses in presbyopic eyes, that are no longer able to accommodate, nevertheless attempt to do so by putting extreme stresses on these lenses and causing



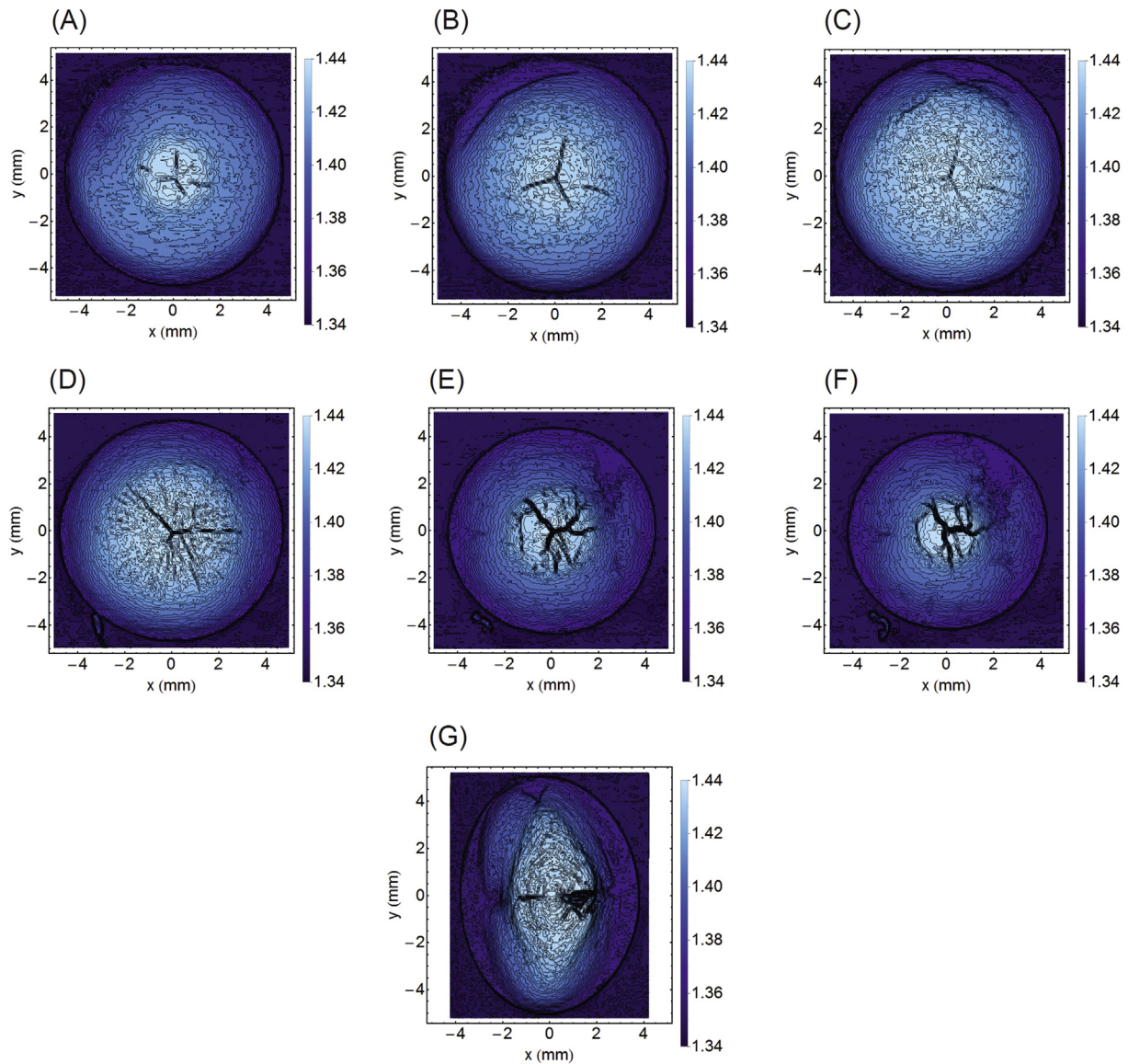
**Fig. 4.** Progression through an 88 year old lens from anterior (A) to posterior (F) poles showing contours of refractive index and areas of high density in Y-shaped branching patterns that alter in different regions of the lens. Each image represents a step of 1.98 mm.

structural disturbances in already weakened layers in these stress prone regions.

The refractive index profiles in the intersecting region between the gradient (cortical) and the plateau (nuclear) sections in Fig. 2D from a lens with a lamellar type irregularity shows the sharp distinction between the gradient and the plateau sections of the profile marked with fluctuations at the intersection. The equatorial profile for this lens (Fig. 3D) does not show these changes as markedly; the irregularities were denser in the polar and surrounding regions than in the equatorial zone unlike the opacities seen by Michael et al. (2008) and are therefore not likely to be linked to mechanical stresses but to other causes. Greater irregularities in the sagittal compared to the equatorial profile may be indicative of the fact that there are more fluctuations, albeit small, in density along the length of the optic axis compared to along the equator. This is because per unit distance along the optic axis, there are more cell layers and a greater number of zones of discontinuity which have been linked to refractive index (Bahrami et al., 2014). With age and the increased predisposition for pathological change, small natural fluctuations in refractive index that initially pose no detriment to vision are more likely to be intensified along the optic axis than in the equator and would have a greater impact on image quality. In Figs. 2 and 3C, representing the sagittal and equatorial profiles of a lens with spoke-like irregularities, both profiles are distorted in shape with fluctuations in the gradient and plateau regions.

Costello et al. (1992) found that although a lens with a mature nuclear cataract had localised cellular disruptions in the form of vacuoles, globules, multi-lamellar and undulating membranes, these were located in the peripheral regions but not in the centre. A lens with a less advanced stage of nuclear opacification manifested very little cellular disruption beyond localised deposition of protein-like material, slightly enlarged extracellular spaces and

minor fluctuations in protein density between adjacent cells. It was concluded that nuclear cataract did not need significant structural disruptions that lead to light scatter in order to attenuate light transmission (Costello et al., 1992). These findings have been supported in subsequent studies (Al-Ghoul et al., 1996; Metlapally et al., 2008; Michael et al., 2008). Nuclear cataract that is caused by light scatter needs to be distinguished from the clinical term for nuclear cataract which manifests as colouration caused by selective absorption of light. The lack of significant cellular damage and the small scale disruptions found in the presence of nuclear scatter (Costello et al., 1992) would be unlikely to produce a detectable fluctuation in refractive index such as seen in this study. Further work is needed to determine how these functional changes can be related to underlying cellular processes. In very advanced forms of nuclear cataract there is a greater degree of damage to fibre cell membranes and more protein-like deposits than in typical or early stage nuclear opacification, creating refractive index fluctuations that could scatter light (Costello et al., 2008). The lenses used in this study were not deemed to have had advanced cataracts in as much as they came from donors in the United Kingdom where an advanced form of opacification such as seen by Costello et al. (2008) would have been treated and replaced by an implant lens. In addition, these lenses did not show a high degree of discolouration. Hence, whilst the changes in the refractive index profiles of these older lenses indicate patterns of abnormality, the measurements are on a macroscopic scale and cannot therefore be directly related to microscopic changes detected in structural studies (Costello et al., 1992; Al-Ghoul et al., 1996; Costello et al., 2008). Nevertheless these are older lenses and many of the structural changes that occur with age (Costello et al., 1992) can, with progression, lead to cataract and indeed some early cataractous changes can be expected in this age group (Vrensen and Willekens, 1989). Disruptions to the refractive index profile are seen in the core (Figs. 1–3A and



**Fig. 5.** Progression through an 85 year old lens from anterior (A) to posterior (F) poles showing contours of refractive index and areas of high density fluctuation in branching patterns that alter in different regions of the lens. The branching pattern becomes complex towards the posterior pole. Each image represents a step of 0.684 mm. (G) The refractive index contours in the sagittal plane show high density fluctuations in the polar region and along the optic axis.

B). They manifest as sharp dips in the central parts of the refractive index profiles (Figs. 2–3A and B) consistent with localised areas of density fluctuation.

Where irregularities in refractive index are in the polar or axial regions (as seen in Fig. 1A and to a lesser extent Fig. 1D), distinct suture-like patterns are evident with progression through the lens (Fig. 4). The characteristics of suture patterns have been well documented and vary in different species in accordance with lens shape and with number of fibre cells which is related to lens size (Kuszak et al., 1994, 2004; Kuszak, 1995). In the human lens, the patterns of sutures range from Y shaped in the layers of the lens laid down in early life to more complex branching in subsequently synthesised cell layers (Kuszak, 1995). In this study, the lenses that had density irregularities in the axial and paraxial regions showed more complex branching in the deeper cortex and nucleus and the simpler Y sutures with progression towards the poles. These patterns, however, indicate regions of refractive index disruptions that are beyond the physiological norm and not merely where the ends

of fibre cells in a healthy lens overlap; the normal sutural pattern would not produce refractive index fluctuations that would be significantly alter the gradient. It is nevertheless interesting that these sutural refractive index fluctuations are less prevalent in outer cortical layers than in the deeper cortex. This could reflect the greater vulnerability of older cells to changes that lead to degeneration and predispose to opacification and/or the higher degree of disorder in older lens layers compared to their younger counterparts (Bassnett et al., 2011). Hence, only relatively minor fluctuations are evident in the fibre cells that form the sutures in the outer cortex. As the branching patterns are caused by rapid fluctuations in refractive index rather than by regions of high density or low density *per se*, it is not possible to determine whether these are caused by an uncommon packing of the cytoskeleton, increased membrane density or changes in protein hydration or deposition. Whilst the lenses in this study are from older donors, it should be noted that advanced age does not necessarily correlate with deterioration of structure: the 86 year old lens in Fig. 1E showed no



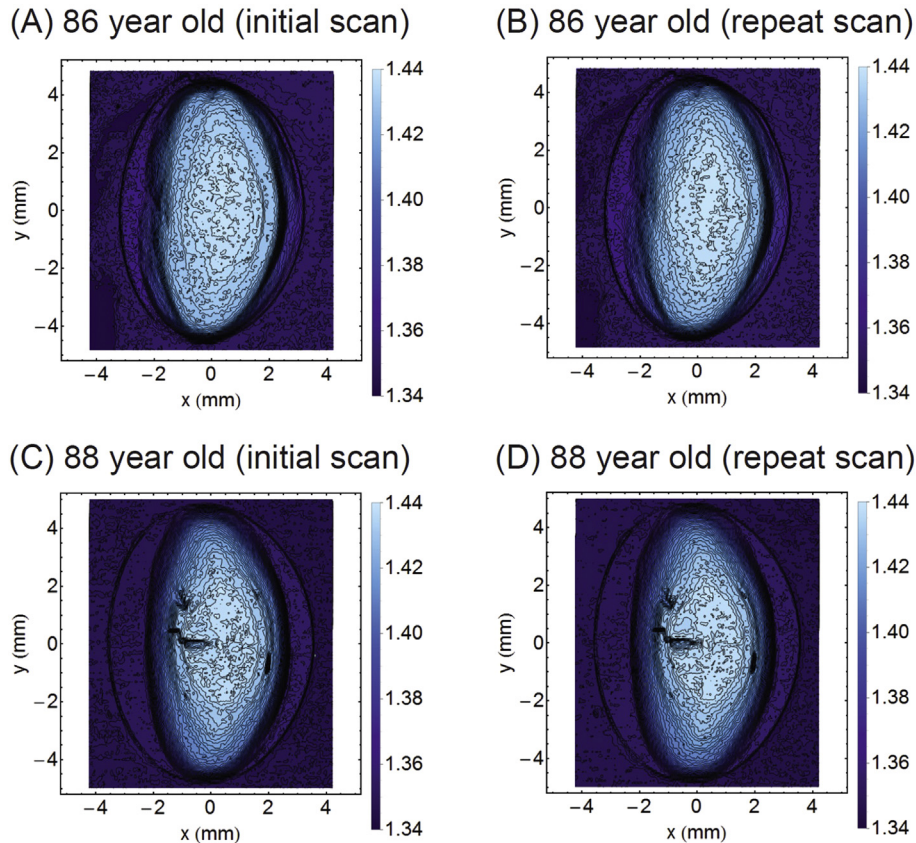


Fig. 6. Refractive index contours in increments of 0.002 for 86 (A and B) and 88 year old (C and D) lenses showing initial (A and C) and repeat (B and D) scans.

evidence of irregularities in refractive index. The contours and profiles in the equatorial and sagittal aspects were undisturbed. The technique used to measure the refractive index gives highly reproducible profiles with no apparent damage resulting in artifacts or experimentally induced opacification from the X-ray source. Lenses had been frozen to allow for transportation and all thawed following the same procedure to minimise any experimentally induced deviations in the thawing process. A previous study that looked at the effects of freezing in both liquid nitrogen and at  $-20\text{ }^{\circ}\text{C}$  on structural order in bovine lenses measured using X-ray diffraction found no alteration in protein order nor permanent structural changes following freezing and thawing using either method (Regini and Meek, 2009). It is highly unlikely that the refractive index disturbances were artifacts caused by freezing and thawing given that the 86 year old lens did not exhibit such changes and these were also not evident in a larger number of lenses from younger age groups, that underwent the same procedure (as yet unpublished). Vrensen and Willekens (1989) in a study on 364 donor lenses in Amsterdam found that around 80% of lenses from the eighth decade of life had some opacities and just under 9% of lenses from the ninth decade were clear. The authors concluded that donor lenses should not necessarily be treated as healthy controls; this applies particularly to older lenses and is to be expected given that age is a major risk factor for senile cataract.

The findings of this study indicate that refractive index irregularities in older lenses have some relation to degenerative changes in structure found in previous studies and shown to be associated with various forms of cataract. Further work to more closely link the breakdown in structure to alterations in the refractive index gradient with age and cataract formation is needed to provide a greater understanding about the relationship between the proteins

and optics of the lens.

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