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Cytoskeletal Alteration and Change of Retinal Nerve Fiber Layer Birefringence in Hypertensive Retina

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Abstract

Purpose: Glaucoma damages the retinal nerve fiber layer (RNFL). Both RNFL thickness and retardance can be used to assess the damage, but birefringence, the ratio of retardance to thickness, is a property of the tissue itself. This study investigated the relationship between axonal cytoskeleton and RNFL birefringence in retinas with hypertensive damage.

Materials and Methods: High intraocular pressure (IOP) was induced unilaterally in rat eyes. RNFL retardance in isolated retinas was measured. Cytostructural organization and bundle thickness were evaluated by confocal imaging of immunohistochemical staining of the cytoskeletal components: microtubules (MTs), F-actin and neurofilaments. Bundles with different appearances of MT stain were studied and their birefringence was calculated at different radii from the optic nerve head (ONH) center.

Results: Forty bundles in eight normal retinas and 37 bundles in 10 treated retinas were examined. In normal retinas the stain of axonal cytoskeleton was approximately uniform within bundles and RNFL birefringence did not change along bundles. In treated retinas elevation of IOP caused non-uniform alteration of axonal cytoskeleton across the retina and distortion of axonal MTs was associated with decreased birefringence. The study further demonstrated that change of RNFL birefringence profiles along bundles can imply altered axonal cytoskeleton, suggesting that ultrastructural change of the RNFL can be inferred from clinical measurements of RNFL birefringence. The study also demonstrated that measuring RNFL birefringence profiles along bundles, instead of at a single location, may provide a more sensitive way to detect axonal ultrastructural change.

Conclusions: Measurement of RNFL birefringence along bundles can provide estimation of cytoskeleton alteration and sensitive detection of glaucomatous damage.

Keywords

Retinal nerve fiber layer; birefringence; cytoskeleton; microtubules; glaucoma

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

INTRODUCTION

The retinal nerve fiber layer (RNFL) in humans consists of bundles of unmyelinated ganglion cell axons. The RNFL exhibits form birefringence due to preferentially oriented ultrastructure.^{1, 2} Two optical techniques have been developed to assess birefringence for detection of RNFL damage in ocular neuropathic diseases, such as glaucoma. Scanning Laser Polarimetry (SLP) measures RNFL retardance, which is proportional to RNFL birefringence and thickness.^{3, 4} Polarization Sensitive Optical Coherence Tomography (PS-OCT) provides simultaneous measurements of RNFL thickness and retardance and then derives RNFL birefringence from the measured thickness and retardance.⁵⁻⁸ Many studies have shown that RNFL retardance of normal retinas varies around the optic nerve head (ONH) and glaucoma causes it to decrease.⁷⁻¹⁶ RNFL birefringence also varies around the ONH in normal retinas and may change in retinas with glaucomatous damage.^{7, 8, 12, 13, 17, 18}

Thus far most clinical studies of RNFL birefringence in glaucomatous eyes evaluate changes of RNFL retardance. RNFL retardance (δ) is the product of RNFL birefringence (n) and thickness (T), that is, $\delta = n \times T$. Retardance is a measure of the birefringence property of the RNFL and can vary with change of either n or T . On the other hand, RNFL birefringence is a tissue property; its value depends critically on details of tissue ultrastructure, but not on tissue thickness. Glaucoma damages retinal ganglion cells (RGCs) and their axons.¹⁹⁻²⁶ Elevation of intraocular pressure (IOP) causes shrinkage of RGC soma, alteration of axonal cytoskeleton and loss of RGCs and axons. Importantly, alteration of axonal ultrastructure can occur before loss of axons.^{21, 22, 25, 26} Hence, measurements of RNFL birefringence may be able to detect early subcellular changes in glaucoma that precede irreversible damage.

The axons of RGCs contain three major cytoskeletal components: microtubules (MTs), F-actin, and neurofilaments (NFs). Several studies demonstrate that axonal MTs contribute to RNFL birefringence. In-vitro treatment with colchicine, an agent that depolymerizes MTs, causes RNFL retardance in rodent retinas to decline nearly to zero.²⁷ In-vivo injection of a solution containing colchicine causes RNFL retardance to decline 20% in primate eyes.²⁸ An electron microscopy study further confirms that MTs are the primary source of RNFL birefringence.²⁹

Although glaucoma damages axonal ultrastructure, little is known about the relationship between changes of RNFL birefringence and its underlying structures in the context of glaucoma. In this study, we used immunohistochemical staining and confocal imaging to evaluate axonal cytoskeleton in retinas with hypertensive damage. The appearance of MTs within bundles was compared with the birefringence of the same bundles to determine if birefringence measurements can reveal the degrees of ultrastructural change.

MATERIALS AND METHODS

Rat Model of Glaucoma

Female Wistar rats, approximately six months old and weighting 250 – 350 g, were used in this study. Animals were housed under a 12-hour light – 12-hour dark cycle with standard food and water provided ad libitum. Experimental glaucoma was induced by translimbal laser photocoagulation of the trabecular meshwork.^{23, 30} The treatment details have been described previously.²³ Laser treatment was administered in the left eye of each rat. Two laser treatments one week apart were applied to maintain elevated IOP of the eyes. The right eye was untreated and served as the control. A rebound tonometer (Tonolab, Colonial Medical Supply, Franconia, NH) was used to monitor IOP after the animals were deeply anesthetized. IOP in both eyes was measured just before treatment and regularly after treatment.

For each eye, mean IOP was calculated as the mean of IOP between the first laser treatment and animal euthanization; peak IOP was the highest IOP value of all measurements from an eye; and cumulative IOP (cIOP) was calculated as the area between the curves of a treated and its contralateral untreated eye in a IOP-versus-day graph.^{24, 25}

Tissue Preparation

After treated eyes were exposed to elevated IOP for about two to three weeks, both eyes of each animal were removed and prepared for optical imaging and immunohistochemical study. Tissue preparation followed previously developed procedures.³¹ Briefly, both eyes of an anesthetized animal were removed and the animal was euthanized. An eye cup of 5 mm diameter that included the optic nerve was excised with a razor blade and placed in a dish of warm (33 – 35°C) oxygenated physiologic solution. After removal of the vitreous, the retina was dissected free of the retinal pigment epithelium and choroid and then draped across a slit in a membrane with the photoreceptor side against the membrane. A second, thinner membrane with a matching slit was put on the RNFL surface to gently flatten the retina. The mounted retina was placed in a chamber perfused with a warm physiologic solution to maintain the tissue in a physiological condition that simulated the retina's *in vivo* state. Tissue preparation, from eyeball excision to a retina placed in the chamber, took about 10 min.

The protocol for the use of animals was approved by the Animal Care and Use Committee of the University of Miami.

Retardance Measurement with a PC'A Polarimeter

An imaging micropolarimeter was used to measure the retardance of RNFL (Figure 1A). The device has been described in detail previously.^{27, 32} Briefly, light from a tungsten-halogen lamp followed by an interference filter (a central wavelength of 500 nm with 10 nm full-width at half maximum) provided monochromatic illumination to an integrating sphere. The beam was then collimated by a lens and incident onto a polarizer (P, a Glan-Thompson polarizing prism). Use of an integrating sphere assured that the output intensity of P varied less than 0.2% as P rotated through 360°. P provided linearly polarized illumination on the

specimen in the chamber. The specimen was then imaged by a lens onto a cooled charge-coupled device (CCD) (Roper Scientific, Tucson, AZ) that provided a pixel size of about 4.5 μm on the specimen in an aqueous medium. The responses were black-level corrected to compensate the dark current and bias level of the CCD. To quantify the retardance of a specimen, a liquid crystal retarder (C') followed by a linear analyzer (A , a Glan-Taylor polarizing prism) was located in the detection arm. A was fixed at 45° . The azimuth and retardance of C' were set to $(0^\circ, 90^\circ)$, $(0^\circ, 200^\circ)$, $(22.5^\circ, 207^\circ)$, and $(-22.5^\circ, 207^\circ)$. Each setting of $C'A$ was characterized by a 1×4 measurement vector D ; the four settings together were characterized by a 4×4 measurement matrix ($\mathbf{D}$) with each row corresponding to one D . $\mathbf{D}$ was determined with a calibration experiment.³² This PC'A system can be used to measure a Stokes vector (S), a 4×1 vector describing the polarization state of a light beam.

Supposing S_{sample} is the Stokes vector of a light beam exiting from a sample, the measured response R_i in an image of the sample can be expressed as

$$R_i = DS_{\text{sample}} \quad (1)$$

where D is the detector vector of $C'A$. With four sets of $C'A$, S_{sample} can be measured as

$$S_{\text{sample}} = \mathbf{D}^{-1}R \quad (2)$$

where $\mathbf{D}^{-1}$ is the inverse of the measurement matrix of $C'A$ and R is a 4×1 response vector corresponding to the four settings of $C'A$.

The PC'A micropolarimeter was used to measure a linear retarder as follows. The Mueller matrix of a linear retarder $\mathbf{M}_{\text{rtd}}$ is a function of its azimuth ρ and retardance δ .^{33, 34} With an incident light of known polarization state S_{in} , the output Stokes vector of a linear retarder is

$$S_{\text{rtd}} = \frac{1}{2}I_0\mathbf{M}_{\text{rtd}}(\rho, \delta)S_{\text{in}}, \quad (3)$$

where I_0 is the incident intensity of the PC'A system. If S_{in} is not an eigenvector of the retarder, Eq. (3) includes four equations with three unknowns I_0 , ρ , and δ . In most cases the system of equations in Eq. (3) was further overdetermined by using more than one S_{in} ; if N known polarization states were used, S_{in} and S_{rtd} became $4 \times N$ matrices. Eq. (3) was solved for I_0 , ρ , and δ by a least-squares fitting.

Measurement and Calculation of RNFL Retardance

To measure RNFL retardance, the polarizer P provided linearly polarized incident light and illuminated the retina with the vitreous side facing the CCD, that is, the beam first passed through the retina and then the RNFL (Figure 1B). For each P position images were taken

with the four settings of C'A described above. Although the isolated retinas were nearly transparent, many birefringent nerve fiber bundles were observable under conditions where the incident polarization state was nearly extinguished by C'A (extinction image). Bundles appeared as either bright or dark stripes (Figure 2).²⁷ Only those observable bundles were analyzed.

The RNFL in transmission was modeled as a linear retarder.^{32, 35, 36} To calculate RNFL retardance, rectangular areas were defined on bundles at the distance (R) from the ONH center of 300 – 700 μm (Figure 2). For each bundle area, the averaged response was calculated and used to derive the output Stokes vector of the area. Because the Stokes vector incident onto the RNFL was modified by the retinal tissue underlying the RNFL, as modeled in Figure 1B, gap areas between nerve fiber bundles were used to estimate the polarization effects of the retina underlying bundles. By assuming a uniform background under the bundles and nearby gaps, the average responses of gap areas were then used to calculate the Stokes vectors of the gap (S_{gap}) with Eqs. (1) and (2). By assuming also that S_{gap} was the Stokes vector incident on the bundles (Figure 1B), the output Stokes vector of a bundle area was expressed as

$$S_{bundle} = K\mathbf{M}_{bundle}S_{gap}, \quad (4)$$

where K is a factor to account for spatial variation in intensity and $\mathbf{M}_{bundle}$ was the Mueller matrix for the bundle. The RNFL was assumed to be a linear retarder, i.e. $\mathbf{M}_{bundle} = \mathbf{M}_{bundle}(\rho, \delta)$. Eq. (4) was solved for ρ and δ as described for Eq. (3). To reduce estimation errors, in experiments P was set at 0° , 45° , 90° and 135° to generate four sets of S_{bundle} and S_{gap} to overdetermine ρ and δ . The units of δ were then converted to optical path length to remove their dependence on wavelength. Note that the same S_{gap} may be applied to all nearby bundle areas.

The effectiveness of using S_{gap} as the Stokes vector incident on bundles was tested by applying S_{gap} to calculate the retardance of the gaps that were defined additionally. The residual retardance of these gaps was less than 0.5 nm, which supported the assumption of a uniform background around the studied bundles.

Confocal Imaging of the RNFL

After optical measurements, a mounted retina was fixed in 4% paraformaldehyde for 30 min at room temperature and rinsed thoroughly in phosphate buffered saline (PBS). The tissue was then removed from the membranes for further immunohistochemical staining and confocal imaging. The detailed procedures have been published previously and are briefly described as follows.²⁶

Three major cytoskeletal components of the axons in a whole mounted retina were simultaneously labeled with phalloidin (1:100, AlexaFluor 488 phalloidin; Invitrogen, Carlsbad, CA) staining F-actin, anti- β -tubulin monoclonal antibody (1:100, Cy3 conjugated;

Sigma-Aldrich, St. Louis, MO) marking MTs and anti-neurofilament antibody (the primary antibody: 1:500, rabbit anti-neurofilament 200 kDa; Sigma-Aldrich; secondary antibody: 1:250 AlexaFluor 647 goat anti-rabbit IgG; Invitrogen) labeling NFs. Nuclei in the inner retina were also identified by 4',6-Diamidino-2-phenylindole (DAPI, FluoroPure grade; Invitrogen) fluorescent counterstain. The fluorescently-stained retina was then imaged by a Confocal Scanning Laser Microscope (cLSM, Leica TCS SP5, Leica Microsystems). The excitation wavelengths for DAPI, AlexaFluor 488, Cy3 and AlexaFluor 647 were 405 nm, 488 nm, 543 nm and 633 nm, respectively. The emission wavelengths detected by the corresponding detectors had the ranges of 415 – 470 nm, 495 – 555 nm, 571 – 621 nm, and 653 – 720 nm. A 40× oil objective provided en face images of a retina with a full field of view of 389 μm × 389 μm and a resolution limited to the sampling density of 0.76 μm/pixel. A three line average scan was used for all the scans. In order to cover all bundles emerging from the ONH, a 3 × 3 tiled array of images was taken with the ONH at the center (Figure 3B), with additional adjacent images added as needed to cover areas measured with the polarimeter. For each array position, en face images were collected at evenly spaced positions in depth (1 μm apart in tissue) starting from the RNFL surface through the retina to a depth at least including the ganglion cell layer. The retina was then reconstructed in 3-D and cross sectional (CS) images were synthesized from the reconstruction with customized software (Figure 3C – F).

Measurement of Bundle Thickness and Calculation of RNFL Birefringence

An en face confocal image of a retina was registered onto the optical image of the same retina by matching the blood vessel patterns (Figures 3A and B).³⁷ A line crossing the bundle area with retardance measurements was drawn in the en face confocal image. The CS images along the line were obtained from the reconstructed 3-D image of the retina. The RNFL was identified by the intensely stained axonal cytoskeleton at the top layer of the retina (Figures 3C – F). To measure the thickness of a bundle, a window centered on the bundle was drawn with its width being approximately half the width of the bundle (dashed lines in Figure 3F). The mean value of T within the window was calculated.

With the measured retardance and thickness of a bundle, the corresponding n , a dimensionless quantity, was then calculated as the ratio of δ and T expressed in units of nm/μm. n was measured along a bundle at different R (Figure 2) to produce a n profile for the bundle.

Evaluation of Axonal Cytoskeleton

To detect cytoskeletal change within bundles, we used a statistical parameter to quantitatively describe the texture of stained nerve fiber bundles in CS images.³⁸ If the intensity in a region has a variance of σ^2 , the parameter $s = 1 - \frac{1}{1 + \sigma^2}$ measures the relative smoothness of the intensity distribution within the region. The parameter s is zero for regions of constant intensity.

To calculate the s of MTs, F-actin and NFs for a bundle, a rectangular box was drawn in the merged CS image of the bundle with the box centered on the bundle and its width being

approximately half the width of the bundle. The box avoided any blood vessels, which were identified by strong phalloidin stain in both CS and en face images. The same box was then applied to the corresponding CS images of MTs, F-actin and NFs, and s for each cytoskeletal component was calculated. In rat retinas MTs are the dominate cytoskeletal component contributing to RNFL birefringence,²⁷ and therefore s of MTs was used to classify cytoskeletal alteration of a bundle.

Statistical Analysis

A linear mixed-model (LMM) analysis accounting for the correlation between multiple measurements made on the same retina and bundles and post hoc least-significant difference (LSD) tests were used to compare measured parameters. The influence of treatment group on the measured parameters was studied with the analysis of covariance formulation of the general linear model (IBM SPSS Statistics 22; IBM Corporation, Somers, NY). The significance level was set at $p < 0.05$.

Image reconstruction and data analysis were implemented with customized software programmed in Matlab (Matlab Version 2012b, The MathWorks, Inc.).

RESULTS

Eight control retinas and 10 treated retinas were studied. The baseline IOP before treatment was not statistically different ($p = 0.56$) with 10.2 ± 0.6 mmHg and 10.0 ± 0.7 mmHg for control and treated eyes, respectively. The IOP of all treated eyes remained elevated for at least two weeks (examples of IOP time course shown in Figures 5B and 6B). Table I summarizes the IOP of the treated eyes. The mean IOP (19.9 ± 3.2 mm Hg) and peak IOP (39.5 ± 7.3 mmHg) of the treated retinas were significantly higher than the control and baseline IOP ($p < 0.0001$), respectively.

Cytoskeleton and Birefringence of Normal RNFL

Examination of en face confocal images showed that all control retinas had approximately uniform stain of cytoskeleton along bundles (Figure 3B). Blood vessels were distinguished from bundles by strong phalloidin staining or as hollow structures. In CS images (Figures 3C – F) bundles were specifically stained, lying just under the retinal surface, and separated from deeper layers by a single layer of nuclei with DAPI counterstain (not shown). Bundle boundaries were sharp and clear.

RNFL birefringence was calculated along bundles. Figures 4A – C show the T , δ and n profiles measured from the bundle areas illustrated in Figure 2. Both T and δ decreased with the increase of distance (R) from the ONH center; the corresponding n , however, varied little. Figures 4D – F summarize the T , δ and n profiles of 40 bundles measured from eight control retinas. These bundles, five bundles from each retina, were randomly selected from different clock-hour sectors of the retinas. Each data point is the mean $\pm$ 2 s.d. (standard deviation) of all bundles that were analyzed at the same R . For each bundle n did not change very much along the bundle with the maximum s.d. of 0.02 nm/ μ m. The average values of n along bundles, however, were significantly different among bundles ($p < 0.0001$), with n ranging from 0.23 – 0.39 nm/ μ m. n was not correlated with T .

Cytoskeleton and RNFL Birefringence in Treated Retinas

With the glaucoma model used in this study, elevation of IOP caused alteration of axonal cytoskeleton. Figures 5 and 6 demonstrate that this alteration was not uniform within a retina. The figures also demonstrate that such a non-uniform alteration could differ among retinas even though the time courses of IOP were similar.

Figure 5A shows the en face image of a treated retina with MT stain. The corresponding IOP time course is shown in Figure 5B. Around the ONH, the appearance of bundles varied from normal-looking (e.g., 10–12 o'clock), distortion (e.g., 6–8 o'clock), to nearly total loss of MTs (5 o'clock). Figures 5C – M illustrate the stain of cytoskeletal components in the CS images of different retinal regions. The CS images along L1 and L2 (Figures 5C – J) show that the stain of axonal MTs, F-actin and NFs was approximately uniform within the bundles, similar to the control. The CS images along L3 (Figures 5K – M) reveal a severely damaged region with loss of nerve fiber bundles. Figure 6 shows another example of non-uniform alteration of cytoskeleton. Although the IOP time course (Figure 6B) of this retina was similar to the retina shown in Figure 5, the en face image demonstrates more extensive damage across the retina. The bundles in the region of L4 show moderate distortion in the en face image; the corresponding CS images (Figures 6C – H) show that the stain was not uniform within bundles. The en face and CS images of L5 (Figures 6F – H) demonstrate severely distorted cytoskeleton within the bundles and bundle boundaries in the MT and F-actin CS images were not clear.

RNFL birefringence was measured for the bundles with different appearances of cytoskeleton alteration. Figure 7 shows the n measured along the bundles (b1 – b5) marked in Figures 5 and 6. The thickness at $R=700\text{ }\mu\text{m}$ for b3 and b4 was measured in additional adjacent confocal images. For all bundles, both T and δ decreased with the increase of R (not shown). Along the normal-looking bundle (b1), the n profile was indistinguishable from normal. Although the appearance of b2 was similar to b1 and its n profile was in the normal range, n at $R=300\text{ }\mu\text{m}$ was lower than the mean -2 s.d of the n at $R=400-700\text{ }\mu\text{m}$, that is, n decreased significantly near the ONH. The n profile along the bundle (b3) located in the severely damaged region was just below the normal range and also approximately constant with R . For the bundle (b4) with moderate alteration, its n profile fell below the normal range and decreased more as it nears the ONH. For the severely damaged bundle (b5), its n decreased significantly at all R ($p < 0.001$).

The Relationship between RNFL Birefringence and MT Texture in Treated Retinas

As shown in Figure 7, it seems likely that change of n can occur early near the ONH. Hence, the texture parameter s of MTs calculated from the CS image at $R=300\text{ }\mu\text{m}$ was used to classify each bundle. Figure 8 displays the s of 40 bundles from eight control retinas and 37 treated bundles from 10 treated retinas. The treated bundles, three to five from each retina, were selected from different retinal regions and include bundles b1 – b5 shown in Figures 5 and 6. The s of the control bundles (circles) appears uniformly distributed, and the Kolmogorov-Smirnov test did not detect significant departure from a uniform distribution ($p = 0.2$). The s values of treated bundles were classified into three groups, depending on their deviation from the mean of the controls ($\bar{s}$). Normal values were defined as less than $\bar{s} + 1.70$

s.d. (the upper 99% tolerance limit for a uniform distribution), moderately abnormal values were defined as those between $\bar{s} + 1.70$ s.d. and $\bar{s} + 4$ s.d, and grossly abnormal values were defined as more than $\bar{s} + 4.0$ s.d. The retinal regions containing the studied bundles and their classified s are listed in Table I. Note that s is zero for regions of uniform intensity. By using these definitions, the s of b1 and b3 was within the normal range, the s of b2 and b4 was moderately abnormal and the s of b5 was grossly abnormal.

Figure 9 shows the grouped n profiles of the 37 treated bundles. For eight bundles with s values within the normal range (excluding five bundles from severely damaged regions, shown as gray diamonds in Fig. 8), seven showed a completely normal n profile, while one showed a n profile within the normal range that decreased as it neared the ONH, that is, n at $R = 300$ μm was lower than the mean $- 2$ s.d. of the n at $R = 400 - 700$ μm (Figure 9A). For the 11 bundles with moderately abnormal s values (Figure 9B), n profiles were either within or below the normal range, but all had decreased n near the ONH. For the 13 bundles with grossly abnormal s (Figure 9C), 11 had n values falling below the normal range and two had n profiles within the normal range but decreased near the ONH, similar to those in Figure 9B.

Among the 37 bundles, five bundles, each from different retinas, were located in severely damaged regions where many bundles had disappeared, as shown with the example of b3 in Figure 5. Interestingly, all five of these bundles had a normal s (gray diamonds in Figure 8). Their n profiles varied from within the normal range to falling just below normal, as shown in Figure 9D.

Using the s of F-actin and NFs to classify the alteration of the same bundles did not provide the well-clustered n profiles achieved by using the s of MTs (data not shown).

Patterns of RNFL Birefringence Profiles in the Treated Retinas

For the treated retinas, unlike the controls, n was not consistently constant along bundles. The examples in Figure 7 demonstrate four patterns of n profiles. To characterize the pattern of a n profile, the measured n was fitted with a three-parameter model, a broken line that slopes upward, then plateaus. The parameters were the slope of the initial segment and the location of the break point. Figure 10 shows the fitting of the n profiles shown in Figure 7. The fits were compared to the mean of the profile (a horizontal line) using an F-test on the residuals of the fit. The n profiles in Figure 10A, C and E were well-fit with their mean values. The n profiles in Figure 10B and D were better fit with a broken line, demonstrating significant decrease near the ONH.

Each of the bundles shown in Figure 9 was tested for decrease near the ONH by fitting with the three-parameter model and setting the p -value of the F-test to 0.15, the value based on best separation of the n profiles grouped in Figure 9A–C. The n profiles were then cataloged into four patterns by the fitted model and by comparing the mean n with the lower range of the normal, that is, 0.23 nm/ μm . The cataloged n profiles are summarized in Table II. For those best fit with a broken line, the lines broke at radii ranging from 344 μm to 500 μm with the mean of 428 ± 56 μm .

DISCUSSION

RNFL birefringence is an intrinsic property of the tissue and, therefore, should depend critically on details of tissue ultrastructure. A question of interest to the clinical application of RNFL birefringence is what a measured n profile would suggest for its underlying ultrastructure. This study aimed to determine if measurements of RNFL birefringence can reveal glaucomatous change of axonal ultrastructure. High IOP in a rat model of glaucoma was used to produce damage to the RNFL that was then examined with imaging micropolarimetry and confocal microscopy. High IOP caused a non-uniform alteration of cytoskeleton across the retina. Birefringence of bundles with different degrees of alteration was measured at various distances from the ONH and compared with the birefringence profiles of normal bundles. We used a texture analysis of cross-sectional confocal images to detect subtle changes of axonal cytoskeleton. Among the bundles examined we found that change of birefringence profiles, either significant reduction of entire profiles or greater reduction near the ONH, was related to the texture change of axonal MTs. Because birefringence is an optical property that can be measured in vivo, the results of this study potentially allow one to infer ultrastructural change from clinical measurements.

In this study, RNFL birefringence was expressed as n , the ratio of bundle retardance in nm and bundle thickness in μm . In normal rat retinas, n of the RNFL was found to be approximately constant along bundles, which suggests that the underlying structure that contributes to RNFL birefringence does not change very much along bundles in normal tissue. Constant RNFL birefringence along bundles is also found in human retinas.^{8, 11, 13, 18} In this study, n in normal retinas varied from 0.23 to 0.39 nm/ μm . This variation is much smaller than that of human RNFL, which has n varying approximately from 0.12 to 0.63 nm/ μm ,^{12, 13, 17, 39} although average n of normal rat and human RNFL are comparable.^{8, 11, 13}

This study used texture analysis of cytoskeletal appearance within the RNFL to demonstrate the relationship between changes of nerve fiber bundle ultrastructure and its corresponding birefringence. Five examples of the variety of cytoskeletal appearances seen in the confocal images of MTs stain are shown in Figures 5 and 6. The images demonstrate that alteration of cytoskeleton varied across the retina and among animals even though the time course of IOP was similar. Texture analysis of MTs in the CS images provided a quantitative measure (s) of ultrastructural change that could differentiate subtle differences between bundles. In Figure 5, for instance, the regions containing L1 and L2 look similar in the confocal images, but the s of MTs showed that the texture of b2 was less uniform than b1 and their corresponding n profiles were also different. We sorted n profiles using the s of MTs based on the knowledge that MTs are the major structure that contributes to RNFL birefringence, knowledge from both in vivo and in vitro studies.^{27–29} On the other hand, we used the goodness of fit of a three-parameter model (broken line) to characterize n profiles and cataloged the n profiles of treated bundles into four patterns as listed in Table II.

Excluding those bundles located in severely damaged regions, which are discussed separately below, n profiles of treated bundles were sorted into three groups depending on the deviation of s values from the mean s of the controls, as shown in Figures 9A – C, and

cataloged into four patterns based on the fitted model and the mean n (Table II). All but one of the 24 bundles that had either moderately or grossly abnormal s of MTs showed a deviated n profile, that is, either decrease of n , greater reduction near the ONH or both. Thus, if birefringence is abnormal, axonal MTs within bundles are abnormal. Figure 9 and Table II also demonstrate that overall change in n increased from the group of bundles with moderate distortion of MTs to that with gross distortion; hence, the appearance of a n profile along bundles might provide an estimate of the distortion of axonal MTs in retinas with different degrees of glaucomatous damage. For those profiles better fitted with a broken line, the break points give the bundle locations where more reduction of n , hence more damage to ultrastructure, occurred. Different from MTs, using the s of F-actin and NFs could not provide well-clustered n profiles, suggesting that there may not be a direct association between the appearance of a n profile and the distortion of F-actin and NFs.

Figure 9D shows a special case, in which the bundles were in severely damaged regions where many bundles had disappeared. The s of these bundles had the normal values and their corresponding n profiles were also within or near the normal range. This result suggests that in severely damaged regions the remaining axons may possess a normal cytoskeleton and may have normal birefringence.

As shown in Figure 9B, pronounced reduction of n occurred near the ONH, suggesting that change of axonal ultrastructure may start early in the portion of a bundle near the ONH. Many studies have investigated topographic change of the RNFL and RGCs with different animal models, with various results. Loss of RGCs and nerve fiber bundles is found to either occur first near the ONH or start peripherally and move toward the ONH.^{40–45} Loss of RGCs and nerve fiber bundles is also found to be either regionally selective or not.^{46–50} These discrepancies in damage features between studies could be due to different animal species and glaucoma models. Note that many of the bundles studied here have experienced minimal loss of cells, but rather exhibit mainly early ultrastructural change, so that this pattern of change may not represent the ultimate pattern of loss.

The two characteristic changes of RNFL birefringence, decrease of n and greater reduction near the ONH, are consistent with our previous findings of changes of RNFL reflectance in hypertensive retinas, that is, decrease of RNFL reflectance occurs in the RNFL with hypertensive damage and first near the ONH.⁵¹ Together, these studies suggest that direct measurement of RNFL birefringence and reflectance and measurement of these properties along bundles, not currently part of clinical practice, can provide estimation of cytoskeleton alteration and sensitive detection of glaucomatous damage and should motivate the development of these optical methods.

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FIGURE 1.

Schematic diagrams of a PC'A system and Stokes vectors of the retina. (A) P: a linear polarizer producing a linearly polarized light; C': a variable retarder; A: a linear analyzer; I_0 : incident intensity; S_{in} : incident Stokes vector on a sample; S_{sample} : output Stokes vector of the sample; M: Muller matrix of the sample; D: a 1×4 vector describing the measurement state of C'A. (B) S_{gap} : Stoke vectors of the light beam exiting from gap areas; S_{gap} is used as the incident Stokes vector of bundles. S_{bundle} : output Stokes vector of a bundle.

FIGURE 2.

Retinal image in transmission with P and C'A set near extinction. A normal retina imaged at 500 nm. Bundles appear either as bright or dark stripes. Retardance is calculated for visible bundles by defining rectangular areas on bundles (black boxes) and nearby gaps (white boxes) between bundles. BV: blood vessel.

FIGURE 3. Confocal images of a whole-mounted normal retina. (A) Retinal image in transmission. (B) Merged en face image of the same retina with MT, F-actin and NF stain. BV: blood vessel. (C – E) Cross sectional images of MT, F-actin and NF stain along the yellow line in B. (F) Merged image of C – E. Dashed lines: the window used for calculation of bundle thickness.

FIGURE 4.

RNFL thickness (T), retardance (δ) and birefringence (n) along bundles in normal retinas. (A – C) T , δ and n profiles along one bundle. Both T and δ decrease with the increase of distance from the ONH center. n , however, is approximately constant along the bundle. (D – F) The mean profiles of 40 bundles from the control retinas. Vertical bars indicate ± 2 s.d. Gray bar in F shows the overall range of normal n . The error bar at the right side of F shows the maximum variation (± 2 s.d.) in n across radii.

FIGURE 5.

Axonal cytoskeleton in a mildly damaged retina. (A) En face image with MT stain showing the variation of bundle appearance across the retina. (B) Intraocular pressure (IOP) time courses. Mean IOP was 16.0 mmHg for the treated eye and 10.13 mmHg for the contralateral untreated eye. Peak IOP was 30 mmHg with cIOP of 108 mmHg-days over 20 days. Vertical lines: standard error of the mean IOP calculated from 10 repeat readings of the tonometer; arrows: the days of the first and second laser treatments. (C – M) CS images along L1, L2, and L3 shown in A. The bundles in L1 and L2 appear normal looking, whereas the retinal region in L3 has lost bundles. b1 – b3: bundles with birefringence analysis in Figure 7.

FIGURE 6.

Axonal cytoskeleton in a moderately damaged retina. (A) En face image with MT stain showing the distortion of bundles across the retina. (B) Intraocular pressure (IOP) time courses. Mean IOP was 15.93 mmHg for the treated eye and 9.89 mmHg for the contralateral untreated eye. Peak IOP was 33 mmHg with cIOP of 117 mmHg-days over 20 days. Vertical lines: standard error of the mean IOP calculated from 10 repeat readings of the tonometer; arrows: the days of the first and second laser treatments. (C – H) CS images along L4 and L5 shown in A. The bundles in L4 show moderate distortion of cytoskeletal components within bundles; the bundles in L5 show apparent non-uniform stain of bundles. b4 and b5: bundles with birefringence analysis in Figure 7.

FIGURE 7.

Birefringence (n) profiles along the bundles b1 – b5 shown in Figures 5 and 6. The n profile indistinguishable from normal (A), in the normal range but decreased near the ONH (B), below the normal range and nearly constant with radius (C, E), and below the normal range and decreased greater near the ONH (D). Gray and error bars: normal range of n and the error range from Figure. 4F.

FIGURE 8.

Texture (s) of MTs in 40 control and 37 treated bundles. Gray circle: the mean of the control ($\bar{s}$). The open and black diamonds are bundles located in either normal-looking or distorted regions, as observed in en face images. The gray diamonds are bundles located in severely damaged regions (see details in Figure 9D and text). b1 – b5 are bundles shown in Figures 5 and 6. Vertical lines mark $\bar{s} + 1.7 \text{ s.d.}$ and $\bar{s} + 4.0 \text{ s.d.}$

FIGURE 9.

n profiles classified by the texture (s) of MTs at radius of 300 μm . (A) Normal s of the bundles shown as open and black diamonds in Figure 8: all n profiles are within the range of the control (gray bar) and for most of the bundles n varies little along bundles. (B) Moderately abnormal s : most of the n profiles are within the normal range and all profiles show decreased n near the ONH. (C) Grossly abnormal s : most of n profiles fall below the normal and a few are within the normal range with a decrease near the ONH. (D) Normal s for the bundles in severely damaged regions (gray diamonds in Figure 8): n profiles are either within or just below the normal range. The number of bundles in A – D is 8, 11, 13 and 5, respectively. Black diamond: mean n ; dashed line: ± 2 s.d. of the measured n . Gray and error bars: normal range of n and the error range from Figure 4 to show the normal variation (± 2 s.d.) in n across radii.

FIGURE 10.

n profiles fitted with a broken line to detect a decrease near the ONH. A fit was compared to the mean of the profile using an F-test on the residuals of the fit. The n profiles in A, C and E were well-fit with the mean. The n profiles in B and D were fit better with a broken lines with break points at (438 μm , 0.29 nm/ μm) and (418 μm , 0.20 nm/ μm), respectively. Open triangles: the measured n , a copy of Figure 7. Dashed lines: the mean of measured n . Heavy lines: fitted lines.

Table I

Summary of the IOP of the treated retinas* and the texture of MTs of studied sectors**

Rat	IOP _{baseline} (mmHg)	IOP _{mean} (mmHg)	IOP _{peak} (mmHg)	cIOP (mmHg-days)	Survival Duration (days)	Texture (s) of MTs			
						Less than $\bar{s} + 1.7$ s.d.	Between $\bar{s} + 1.7$ s.d. and $\bar{s} + 4$ s.d.	Greater than $\bar{s}$ + 4 s.d.	Less than $\bar{s} + 1.7$ s.d. (for sectors with severe damage)
1	10	14	31	36	16		S9, S11, S12	S8	
2	10	16	32	59	16	S5, S10	S3		
3	10	22	30	108	20	S9	S11		S4
4	9	21	44	114	20		S5	S8, S10	S3
5	10	20	47	116	21	S6, S8	S1	S2	
6	9	18	33	117	20		S3, S5	S10	
7	10	22	46	120	20	S7		S2, S5	S11
8	11	19	39	122	21	S9	S10	S1, S3	
9	11	23	48	127	21	S7	S8	S1, S12	S2
10	10	24	45	135	21			S1, S7	S12
Mean ± s.d.	10.0 ± 0.6	19.9 ± 3.2	39.5 ± 7.3	105.4 ± 31.9	19.6 ± 2.0				

* IOP_{baseline} was measured just before the first laser treatment; IOP_{mean} was the mean of the IOP measured at a survival duration, which was defined as the days between the first laser treatment and animal euthanasia for optical measurements; IOP_{peak} was the highest IOP value of all measurements from an eye; cIOP, cumulative IOP, was calculated as the area between the curves of the treated and the contralateral control eyes in a IOP-versus-day graph.^{23, 30} The table is ordered by the values of cIOP from lowest to highest.

** Bundle location, where S with a number denotes approximate clock hour around the ONH. Retinal orientation is clockwise from dorsal, caudal, and ventral to rostral regions with dorsal assigned as 12 o'clock. $\bar{s}$ is the mean s of the control bundles; see Figure 8 and details in the section Cytoskeleton and RNFL Birefringence in Treated Retinas.

Table IIPatterns of n profiles for the bundles with different MT texture (s)

	Eight bundles with normal s (Figure 9A)	Eleven bundles with moderately abnormal s (Figure 9B)	Thirteen bundles with grossly abnormal s (Figure 9C)	Five severely damaged bundles with normal s (Figure 9D)
n profile indistinguishable from normal	7		1	4
n profile in the normal range but decreases near the ONH	1	9	1	
n profile below the normal range and decreases near the ONH		2	6	
n profile below the normal range and constant with radius			5	1