The Relationship between Retinal Ganglion Cell Axon Constituents and Retinal Nerve Fiber Layer Birefringence in the Primate

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PURPOSE. To determine the degree of correlation between spatial characteristics of the retinal nerve fiber layer (RNFL) birefringence (Δn(RNFL)) surrounding the optic nerve head (ONH) with the corresponding anatomy of retinal ganglion cell (RGC) axons and their respective organelles.

METHODS. RNFL phase retardation per unit depth (PR/UD, proportional to Δn(RNFL)) was measured in two cynomolgus monkeys by enhanced polarization-sensitive optical coherence tomography (EPS-OCT). The monkeys were perfused with glutaraldehyde and the eyes were enucleated and prepared for transmission electron microscopy (TEM) histologic analysis. Morphologic measurements from TEM images were used to estimate neurotubule density (nR(E)), axoplasmic area (Aa) mode, axon area (Aa) mode, slope (m) of the number of neurotubules versus axoplasmic area (neurotubule packing density), fractional area of axoplasm in the nerve fiber bundle (f), mitochondrial fractional area in the nerve fiber bundle (xm), mitochondria-containing axon profile fraction (m), and length of axonal membrane profiles per unit of nerve fiber bundle area (Lmem/Aa). Registered PR/UD and morphologic parameters from corresponding angular sections were then correlated by using Pearson’s correlation and multilevel models.

RESULTS. In one eye there was a statistically significant correlation between PR/UD and nR(E) (r = 0.67, P = 0.005) and between PR/UD and neurotubule packing density (r = 0.70, P = 0.002). Correlation coefficients of r = 0.81 (P = 0.01) and r = 0.50 (P = 0.05) were observed between the PR/UD and Aa modes for each respective subject.

CONCLUSIONS. Neurotubules are the primary source of birefringence in the RNFL of the primate retina. (Invest Ophthalmol Vis Sci. 2009;50:5238–5246) DOI:10.1167/iovs.08-3263

GLAUCOMA is a progressive optic nerve disease characterized by a loss of retinal ganglion cell (RGC) axons and thinning of the retinal nerve fiber layer (RNFL). Axon loss can be as much as 50% before the disease is clinically detectable.1 Imaging devices have been developed to quantitatively measure RNFL thinning and birefringence loss. For example, optical coherence tomography (OCT) produces cross-sectional images of the retina allowing measurement of RNFL thickness.2 Polarization-sensitive OCT (PS-OCT)3 and scanning laser polarimetry (GDx; Carl Zeiss Meditec, Inc. Dublin, CA)4 measure phase retardation in the RNFL that arises from anisotropic structures in the RNFL. Changes in RNFL birefringence may precede RGC death.

Birefringence (Δn) is a dimensionless measure of the anisotropy of the refractive index (nR(E)) of a material and is given by the difference between the extraordinary index (nR(E)) and ordinary index (nR(O)), where nR(E) and nR(O) are the refractive indices for polarization perpendicular and parallel to the axis of anisotropy respectively (Δn = nR(E) - nR(O)). Phase retardation is proportional to birefringence according to the expression Δn = λp/PR × ΔZ where λp is the free-space wavelength, PR is the phase retardation, and ΔZ is the thickness of the material.5 In general, Δn of the RNFL is weakly wavelength dependent across visible and near infrared wavelengths.7 The RNFL demonstrates form birefringence that originates from anisotropic cylindrically shaped cellular structures in the RGC axons.8,9 The major cylindrical structures of the RGC axonal cytoskeleton are neurotubules (NT), neurofilaments, and neurotubule-associated proteins.10 Huang and Knighton5 identify neurotubules as the source of birefringence from the RNFL. Theoretical analysis has attributed RNFL reflectance to the cylindrically shaped mitochondria and axonal membranes.9

It is postulated that either changes in neurotubule density of RNFL bundles or neurotubule packing densities within the axons themselves are responsible for the differences in birefringence surrounding the ONH.11 Neurotubule density (nR(E)) is defined in this report as a scalar estimate of the number of neurotubules per unit area of RNFL tissue (NT/μm²). There is strong evidence that the regional characteristic nR(E) surrounding the optic nerve head (ONH) is a source of birefringence5,12,13 but it has not been clear whether nR(E) is the only source of birefringence.9 RNFL birefringence has been investigated as a possible diagnostic to detect early subcellular changes in glaucoma before there is any measurable change in RNFL thickness.5,12,13 Fortune et al.14 injected colchicine into the vitreous of nonhuman primate eyes and observed neurotubule disruption with a reduction of RNFL birefringence without any accompanying change in RNFL thickness. These results show that decreases in the number of neurotubules occur before thinning of the RNFL, and in situ measurement of nR(E) can be used as a diagnostic for cytoskeletal changes in RGC axons.15 In this study, we compared the measured birefringence signal in the peripapillary retina with the corresponding measured anatomic features to explore the origin of the RNFL birefringence.
METHODS
Measurement of RNFL Birefringence

The retinas of two healthy 6-year-old female, 5.9 and 7.7 kg, cynomolgus monkeys (subjects 1853 and 57204) were imaged over a period of 62 days. Intraocular pressures and subjective visual function were normal. All experimental procedures were approved by The University of Texas at Austin Institutional Animal Care and Use Committee (IACUC, Protocol 05021401) and conformed to all United States Department of Agriculture (USDA), National Institutes of Health (NIH) guidelines, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A combination of IM ketamine (10 mg/kg) and xylazine (0.25 mg/kg) was used to anesthetize the monkeys, and anesthesia levels were monitored by a certified veterinary technologist. A retrobulbar injection of 0.5% xylocaine and 1 drop of 1% tropicamide. One drop of 10% methylcellulose, to prevent dehydration, was placed in the eye to be imaged, and a contact lens was placed on that eye. A contact lens that rendered the monkey slightly myopic was used to ensure that light was focused on the internal limiting membrane (ILM).

The peripapillary RNFL thickness (Z\textsubscript{RNFL}) and single-pass phase retardation maps of the two cynomolgus monkeys were measured by using an enhanced polarization-sensitive optical coherence tomography (EPS-OCT) system described previously\textsuperscript{11,14}. EPS-OCT consists of a PS-OCT instrument combined with a nonlinear fitting algorithm to determine PR and \( \Delta n \) with high sensitivity in weakly birefringent tissues. The system utilizes a modelocked Ti:Al\textsubscript{2}O\textsubscript{3} laser source (\( \lambda_0 = 830 \text{ nm}, \Delta \lambda = 55 \text{ nm} \)) linearly polarized at 45°. The Z\textsubscript{RNFL} and PR maps are used to construct phase retardation per unit depth (PR/UD) area maps given in units of degrees of retardation per 100 \( \mu \text{m} \) of RNFL thickness (degrees/per 100 \( \mu \text{m} \)) by dividing local PR by Z\textsubscript{RNFL}.

The PR map was measured in an area and ring scan configuration. Each area map consisted of 24 evenly spaced (15°) radial scans containing 2.6, or 8 clusters distributed uniformly between 1.4 and 1.9 mm from the center of the optic nerve head. A cluster comprised 36 or 64 A-scans individually acquired over a respective 0.9- or 1.6-mm\textsuperscript{2} area. Ring scans are derived from sector scans spread 5° apart. The right eye of each subject was imaged twice on separate days, to assess reproducibility of retinal birefringence measurements.

A single peripapillary map was acquired in approximately 45 minutes. Laser power incident on the cornea was 2.8 mW during lateral scanning and 1.7 mW while stationary for both scan configurations. Approximate laser spot size at the retinal surface was 30 \( \mu \text{m} \). Axial resolution was 5 \( \mu \text{m} \) (determined by the coherence length of the laser source in air).

RGC Axon Organelle Sampling

The PR/UD sinuous pattern\textsuperscript{11,15,16} surrounding the ONH was used to determine the angular interval around the ONH that RGC axon organelles must be sampled. Using the Nyquist criterion, RGC axon organelles were measured in eight angular sections (octants) of the peripapillary retina in the first eye sampled (1853 OS). Based on the correlation result between birefringence and \( \phi \text{norm} \), the first eye of the first subject was imaged to determine the angular interval section should be sampled around the ONH of the second eye (decreasing bundle variance within a region) to increase the power to 80% and detect a significant correlation between \( \phi \text{norm} \) and birefringence (Fig. 1). Each bundle within an angular section was chosen at random to eliminate bias. Nerve bundles were sampled from a 0.15-mm\textsuperscript{2} rectangular area from each angular section, which contained approximately 15 to 20 nerve fiber bundles; therefore, 3 nerve fiber bundles approximately 15% to 20% and 7.5% to 10% of the sampled area of subjects 57204 and 1853, respectively. The exact number of bundles within each angular section was not known.

Preparation of Ocular Tissue

Two days after EPS-OCT imaging was completed, both monkeys were anesthetized and transcardially perfused with a fixative solution (pH 7.4) consisting of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer. Each animal was first brought down to a surgical level of anesthesia with a combination of IM ketamine (10 mg/kg) and xylazine (0.5 mg/kg). Pentobarbital sodium (10 mg/kg) was given first for surgical anesthesia and then an IV overdose was administered.

After each perfusion was complete, all four eyes were enucleated and immersed in 180 mL of the primary fixative solution from 1 to 3 hours before dissection from the periorbital tissue and removal of the posterior hemisphere of the eye. A 360° incision was made at the ora serrata, and the anterior eye structures including the ciliary body, iris, lens, and cornea were removed from the posterior eye cup. A notch and suture placed in the nasal sclera before enucleation remained visible throughout the specimen preparation process to maintain proper orientation. All posterior eye cups were then simultaneously immersed in 180 mL of the primary fixative solution for approximately 1.5 hours.

Eye cups were washed in three 30-minute changes of 0.1 M sodium phosphate buffer (180 mL each, pH 7.4) and postfixed in 90 mL of 2% osmium tetroxide (aqueous) solution for 1 hour. They were washed again two times for 30 minutes per wash in 0.1 M sodium phosphate buffer (180 mL each, pH 7.4) and then one more time in 180 mL of distilled water for 30 minutes. The eye cups were then dehydrated
through a series of increasing ethanol concentration solutions (50% for 14 hours, 75% for 1 hour, 100% for 1 hour, and 100% for 1 hour, all in 180 mL) followed by two immersions in acetone (50 mL 100% acetone for 1 hour each). The posterior eye cups were then infused with 1:3 low-viscosity resin (EMBed-812; Electron Microscopy Sciences, Hatfield, PA) in acetone for 1 hour, after which they were covered and left in 2:3 resin for 19 hours overnight. The following day, the eye cups were placed into fresh solutions of 100% resin and left in uncovered containers for approximately 5 hours. They were then placed anterior side down into cubical embedding molds. The molds were put into a 60°C oven for 21 hours overnight, during which the resin solution polymerized, and the eye cups were removed the following day.

Superficial lines intersecting at the center of the ONH profile were used to divide the cube mold of the embedded eye into wedge-shaped angular sections. Each sample wedge was carefully carved from the cube to prevent excess tissue removal surrounding the ONH. Glass knives were used to create a flat rectangular block face (500 × 300 μm).

Transmission Electron Microscopy

Ultrathin and semithin sections were sampled 1.6 to 1.9 mm from the ONH in regions (Ultracut UCT Ultramicrotome; Leica Microsystems GmbH, Ernst-Leitz-Strasse, Germany). Semithin sections (0.5 μm) were cut and stained with toluidine blue before ultrathin sections (60 nm) to ensure that the block’s orientation on the microtome would result in transverse cuts. Several ultrathin sections were placed on copper, α-numeric–indexed grids (Electron Microscopy Sciences) and stained with 2% uranium acetate and 0.3% lead citrate before TEM analysis.

Gray-scale digital images were captured on a transmission electron microscope (EM208 TEM; Phillips, Eindhoven, The Netherlands) equipped with a digital camera system (Advantage HR 1 MB; AMT, Danvers, MA). Each bundle was photographed at low (2,200×–2,800×), medium (11,000×–14,000×), and high (28,000×–44,000×) magnification.

Identification of RGC Axons and Organelles

All morphologic measurements were made using Image J (ver. 1.36; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) on a laptop computer (Hewlett Packard Compaq, Palo Alto, CA). Low magnification TEM images were used to measure nerve fiber bundle area (A<b>0</b>; Fig. 2).

A montage of each nerve fiber bundle was created from a series of medium-magnification images (Photoshop, ver. 8.0; Adobe Systems, San Jose, CA; Fig. 3). RGC axons were captured in high-magnification images to measure respective axonal cross-sectional area (A<b>k</b>) and neurotubule number (k), mitochondria area (A<b>N</b>), number of nontubular organelles (A<b>A</b>), and unidentifiable features of the RNFL bundle. Neurotubules in each of the selected RGC axons were manually counted using the Cell Counter plug-in feature in Image J. A single person identified and counted the RGC organelles in subject 1855 and two different people identified and counted organelles for subject 57204.

The RNFL anterior and posterior boundaries were defined as the ILM vitreous interface and the anterior-most RGC bodies, respectively. If RGC bodies were not present directly posterior to the nerve fiber bundle, then the posterior boundary was defined at the points where RGC axons were absent. A line that bisected Müller cell processes and their footplates at the ILM defined boundaries between adjacent nerve fiber bundles. Axons that appeared pale and had substantially fewer neurotubules were identified as “sparse” axons and were recorded apart from the “nonsparse” axons (Fig. 4). Amacrine cells or glial cells were not included in RGC axons counts when criteria described previously were used.18

Neurotubule Density Estimation

Non-Sparse Axons. RGC axoplasmic area (A<b>x</b>) measurements from TEM images were used in a statistical algorithm to estimate ρ<sub>RNFL</sub> (A<b>x</b>) was calculated as the difference between (A<b>x</b>) and the total organelle area (A<b>m</b> and A<b>n</b>) for each RGC axon and was then plotted against the respective number of neurotubules (k) in a scatter plot (Excel; Microsoft, Redmond, WA) to determine k(A<b>x</b>). Axoplasmic area probability distribution functions (p(A<b>x</b>)) were then computed for each bundle using a kernel estimation method based on an Epanechnikov kernel function. A Freedman-Diaconis bin width rule was used to determine the bandwidth of the kernel smoothing window and was adjusted using Scott’s skewness factor. The axoplasmic fractional area in the bundle (f<sub>x</sub>) is determined by summing all A<b>x</b> measurements in the bundle and then dividing the result by the area of the bundle (A<b>x</b>):

\[

t_x = \frac{\sum_{i=1}^{N} A_{x_i}}{A_x}
\]

Calculations of k(A<x>), p(A<x>), and f<sub>x</sub> are used to estimate RNFL neurotubule density in the nonsparse axons of the nerve fiber bundle according to the expression:

\[
\rho_{RNFL} = f_x \int p(A_x) \frac{k(A_x)}{A_x} dA_x
\]

where ρ<sub>RNFL</sub> is given in units of number of neurotubules per unit RNFL area.

The right side of equation 2 is essentially a twice-weighted sum of RGC axoplasmic neurotubule densities.

\[
\rho_s(A_x) = \frac{k(A_x)}{A_x}
\]

which are estimated by the k(A<sub>x</sub>) linear regression model. Individual estimates of neurotubule counts for each axon (ρ<sub>s</sub>) are weighted by the probability of a given A<sub>x</sub> in the nerve fiber bundle (p(A<sub>x</sub>) dA<sub>x</sub>) and by the fraction of axoplasmic areas in the bundle (f<sub>x</sub>) which accounts for
null $\rho_{\text{RNFL}}$ values in non-RGC areas of the RNFL bundle and effectively normalizes $\rho_x$ into $\rho_{\text{RNFL}}$.

**Sparse Axons.** A different statistical method is used to estimate $\rho_{\text{RNFL}}$ in the temporal maculopapillary fibers (angular sections $T_3$, $T_4$, and $T_5$) for both subjects. The $A_x$ range for the nonsparse axons in this region was a narrow range and resulted in a low $R^2$ in linear fits of $k$ versus $A_x$.

One hundred nonsparse axons were selected from the angular regions. The morphologic characteristics of the selected axons resembled nonsparse axons in other regions of the eye. The sample set was used to generate an average nonsparse axoplasmic neurotubule density ($\rho_{\text{ax}}$) from which a 25% neurotubule density threshold was set to classify sparse axons in the respective region. Once sparse axons were identified, a sparse axon neurotubule density ($\rho_{\text{ax}}$) was determined. The $\rho_{\text{ax}}$ and $\rho_{\text{ax}}$ were individually weighted by their respective $f_x$ and summed to get an estimate for $\rho_{\text{RNFL}}$ in the respective section according to:

$$\rho_{\text{RNFL}} = f_{x,\text{ns}} \cdot \rho_{x,\text{ns}} + f_{x,s} \cdot \rho_{x,s}$$

### Experimental Correlation between Birefringence and RGC Organelles

Radial area and ring scan $PR/UD$ measurements falling within an angular section were averaged, resulting in an equal number of $PR/UD$ values for every mean $\rho_{\text{RNFL}}$. The averaged $PR/UD$ measurements for subjects 1853 and 57204 resulted in $\pm 22.5^\circ$ and $\pm 11.25^\circ$ of angular error, respectively. Average $PR/UD$ measurement positions were optimally registered with angular RGC axon organelle mean measurements to compute the correlation between them (Fig. 5). The shift was used to avoid $PR/UD$ and $\rho_{\text{RNFL}}$ measurement positions taken at angular boundaries between sections in both subjects.

Two approaches were used to find a correlation between $PR/UD$ and RGC organelles. The first approach was a Pearson product moment correlation. Correlation coefficients and significance values were computed (SAS ver. 9; SAS, Cary, NC). A multilevel modeling technique was used for the second approach, since it allows $PR/UD$ and RGC organelles to be correlated on two levels so that finer angular resolution of $PR/UD$ measurements could be preserved. Multilevel modeling was implemented through the procedure PROC MIXED within the statistical analysis software. The model accounts for the random effect of angular position of $PR/UD$ measurements.

### Results

#### Primate RNFL Birefringence

Spatially resolved RNFL peripapillary $PR/UD$ maps were obtained by EPS-OCT from four eyes of two subjects. Results from both subjects are presented throughout the results section to demonstrate the similarities and differences in birefringence and morphologic measurements.

Radial area and ring scan measurements of $PR/UD$ were relatively higher in the superior and inferior areas in both subjects (Fig. 6). Clusters of $PR/UD$ radial area and ring scans within respective $\rho_{\text{RNFL}}$ angular section boundaries were aver-
aged, resulting in an equal number of PR/UD measurements for every RNFL value.

The PR/UD radial area and ring measurements are grouped into regional quadrants and averaged to give the mean PR/UD values listed in Table 1. One-way ANOVA was used to compare quadrant means. The temporal and inferior pair was the only quadrant pair that was not significantly different (P > 0.05) for subject 1853. All quadrant pairs excluding the inferior and nasal pair were significantly different (P < 0.05) for subject 57204.

PR/UD measurement reproducibility was determined for both radial area scans and the ring scan of subject 57204 by calculating the standard error (SE) for each of the PR/UD measurements that were averaged within pRNFL angular section boundaries. To calculate the SE for each PR/UD measurement, the standard deviation (SD) of the cluster PR/UD measurements within angular section boundaries were divided by the square root of the number of clusters. The highest SE of all PR/UD measurements was 7.1 deg/100 μm in the nasal region. The average SE of all three scans was 1.9 deg/100 μm (0.4 ± 10^-3) and was calculated by dividing the average SD of cluster PR/UD measurements from all three scans by the square root of the number of scans.

Primate RNFL Neurotubule Density

Estimates of pRNFL for all bundles in both eyes are given in Tables 2 and 3 and are shown graphically in Figure 7A. Neurtubule densities were highest in the superior and inferior quadrants and lowest in the temporal and nasal regions of both subjects. The Pearson product moment correlation was used to find the similarity between the octant mean pRNFL between the two subjects (Fig. 7B). The result was a correlation coefficient of 0.97 (P < 0.001).

The linear relationship between k and Ax used in the pRNFL estimate was similar across all angular sections for both subjects (Fig. 8A). As axon size increased, the neurotubule number also increased, but the number of neurotubules per unit axon area decreased. This finding is similar to other studies investigating axon size and neurotubule density in the optic nerve head.19

Correlation between Birefringence and RGC Organelles

Subject 57204 had a correlation coefficient of 0.67 (P = 0.005) between PR/UD and pRNFL (Table 4), and the multilevel model resulted in standardized regression coefficients of 0.50 (P = 0.04). The averaged pattern of PR/UD is shown superimposed on respective pRNFL for both subjects in Figure 9.
Morphologic measurements from TEM images were used to estimate mean values of axoplasmic area (A_p) mode, axon area (A_s) mode, slope (\(\omega\)) of neurotubule number versus axoplasmic area (neurotubule packing density), fractional area of axoplasm in the nerve fiber bundle (f_A), mitochondrial fractional area in the nerve fiber bundle (c_m), mitochondria-containing axon profile fraction (\(m_p\)), and length of axonal membrane profiles per unit nerve fiber bundle (L_m/A_s).

Pearson product moment correlation coefficients of the morphologic parameters with PR/UD are summarized in Table 5. Correlation coefficients of \(r = 0.81\) (P = 0.01) and \(r = 0.5\) (P = 0.05) were observed between PR/UD and mean \(A_s\) mode in subjects 1853 and 57204 (Fig. 10), respectively. Mean \(A_s\) mode values were similar in the superior and inferior portions of both eyes and were larger than temporal and nasal portions. Mean \(A_s\) and PR/UD yielded a significant correlation coefficient of \(r = 0.79\) (P = 0.01) in subject 1853 (Fig. 10B).

In subject 57204, a significant correlation coefficient of \(r = 0.7\) (P = 0.002) was observed between section averaged PR/UD and the \(k\) versus \(A_s\) regression fit slope (\(\omega\)) that is used as a measure of neurotubule packing density (Fig. 11). Arcuate bundle regions had the highest packing density, followed by the nasal region, and last the papillomacular region.

**Discussion**

**Primate RNFL Birefringence**

PR/UD measurements were highest superior and inferior to the ONH for both subjects. In subject 57204, the average of the quadrant PR/UD values given in Table 1 for the superior and temporal region are 18.2 ± 1.0 deg/100 um (\(\Delta n = 4.2 \times 10^{-4}\)) and 9.3 ± 0.3 deg/100 um (\(\Delta n = 2.1 \times 10^{-4}\)). Cense et al. measured the RNFL birefringence of two human subjects in vivo using PS-OCT at 840 nm. They also reported higher RNFL birefringence (4.1 \(\times 10^{-4}\) in the superior and inferior areas with the lowest in the temporal region (1.2 \(\times 10^{-4}\)). Huang et al. calculated similar results of averaged birefringence for the superior (4.2 \(\times 10^{-4}\)) and temporal region (2.5 \(\times 10^{-4}\)) of 12 human eyes. The SE was greatest in the nasal region for subject 57204 and may have resulted from a thinner RNFL area or eye movements. This finding was similar to others.

Uncertainty in the PR/UD measurements comes from any error in the RNFL thickness (\(Z_{RNFL}\)) measured from EPS-OCT intensity images and phase retardation (PR) estimates from the EPS-OCT algorithm. Error in the \(Z_{RNFL}\) comes from the refractive index range for the RNFL (4%, \(n = 1.34–1.39\)) and uncertainty in the exact location of the RNFL boundaries due to the axial resolution limit of the EPS-OCT instrument (5 \(\mu m\)). Noise creates an uncertainty of approximately ±1° for PR estimates. Thus, uncertainty in PR is fractionally greater in regions with low PR such as the nasal and temporal regions. Radial and rotational error in position of the eye during imaging may have caused a registration error in PR/UD measurements.

**Neurotubule Density of the Primate RNFL**

Sources of uncertainty for \(\rho_{RNFL}\) determination include: sampling RNFL bundles near the angular section boundaries, misclassified cells or cellular structures, different observers for the two eyes studied, and sampling location from the ONH for each eye. The higher \(\rho_{RNFL}\) values for subject 1853 can be explained by counter bias between the first and second eye. The mean difference (95% limits of agreement) between neurotubule counts of the same RGC axons of two counters for subject 57204 was –1.6(−8.8 to 5.6) neurotubules.

Some angular sections had more variability in \(\rho_{RNFL}\) within nerve bundles than others. Most of the variability could be attributed to differences in RNFL nerve bundle area (\(A_s\)) measurements, since \(\rho_{RNFL}\) estimates are calculated using \(A_s\). The standard deviation of RNFL thickness of human retina ranges from 15 \(\mu m\) in the temporal region to 26.5 \(\mu m\) in the inferior

| Table 1. PR/UD (Proportional to Birefringence) Quadrant Means of Radial Area Scan and Ring Scan Measurements in One Eye of Each Subject |
|----------------|----------------|----------------|----------------|----------------|
| Section        | Mean            | SD             | Mean            | SD             |
| Superior       | 19.1            | 2              | 18.3            | 2.6            |
| Inferior       | 14.1            | 3.1            | 14.9            | 2.6            |
| Temporal       | 9.4             | 3.3            | 9.0             | 3.3            |
| Nasal          | 12.5            | 4.5            | 15.5            | 12.4           |

| Table 2. Estimated Neurotubule Densities in Three Nerve Fiber Bundles of Each RNFL Octant |
|----------------|----------------|----------------|----------------|----------------|
| Section        | Bundle 1       | Bundle 2       | Bundle 3       | Mean           | SD             |
| NS             | 66.0           | 72.2           | 76.3           | 71.5           | 5.2            |
| SS             | 110.1          | 69.8           | 64.6           | 81.5           | 24.9           |
| ST             | 126.5          | 79.4           | 85.4           | 97.1           | 25.7           |
| TS             | 69.9           | 64.7           | 69.6           | 68.1           | 2.9            |
| TL             | 32.5           | 28.8           | 37.1           | 33.0           | 4.4            |
| IT             | 80.5           | 96.3           | 69.9           | 82.2           | 13.3           |
| LT             | 98.4           | 77.0           | 68.3           | 81.2           | 15.5           |
| NI             | 49.6           | 74.6           | 90.1           | 71.4           | 20.4           |

| Table 3. Estimated Neurotubule Densities in Three Nerve Fiber Bundles of Each RNFL Section |
|----------------|----------------|----------------|----------------|----------------|
| Section        | Bundle 1       | Bundle 2       | Bundle 3       | Mean           | SD             |
| NS             | 53.7           | 42.6           | 37.1           | 44.5           | 8.5            |
| SS             | 66.9           | 54.2           | 36.1           | 52.4           | 15.5           |
| ST             | 77.0           | 45.1           | 68.4           | 63.5           | 16.5           |
| TS             | 87.7           | 66.9           | 68.3           | 74.3           | 11.6           |
| TL             | 45.1           | 59.8           | 45.8           | 50.2           | 8.8            |
| IT             | 63.0           | 18.1           | 60.8           | 47.3           | 25.3           |
| LT             | 33.0           | 26.2           | 33.5           | 30.9           | 4.1            |
| NI             | 4.5            | 9.3            | 33.1           | 15.6           | 15.3           |
| NS             | 11.3           | 5.6            | 3.7            | 6.9            | 3.9            |
| SS             | 46.5           | 35.0           | 42.3           | 41.3           | 5.8            |
| ST             | 72.6           | 24.5           | 83.5           | 60.2           | 31.4           |
| TS             | 50.6           | 65.6           | 47.2           | 54.5           | 9.8            |
| TL             | 42.1           | 75.7           | 41.4           | 52.4           | 18.5           |
| IT             | 73.7           | 22.3           | 25.6           | 40.5           | 28.8           |
| LT             | 18.4           | 73.0           | 76.7           | 56.0           | 32.6           |
| NI             | 17.7           | 42.0           | 38.8           | 32.9           | 13.2           |
Standard deviations of RNFL greater than 20 NT/μm² occurred in the arcuate bundles and the bundles located in the inferior nasal portion of the RNFL near major blood vessels. Bundles lying close or adjacent to temporal blood vessels that approximately overlapped the arcuate nerve bundles were not excluded from the sampled population. The few histologic studies of primate RNFL thickness did not measure thickness near large blood vessels because RNFL borders could not be accurately determined. The RNFL bundle area variation near and away from blood vessels as well judgment as to the location of the RNFL borders would produce variability in RNFL estimates.

Glial content (Müller cells and astrocytes) of nerve bundles would also contribute to bundle area variation seen within the same angular section since glial tissue is included in the Aₙ measurement. Ogden reported that the proportion of RNFL bundle area occupied by glia is independent of RNFL thickness and does not vary regularly with distance from the ONH in cynomolgus and rhesus monkeys. Angular sections with the highest variability in RNFL estimates also had the largest variability in glial content percentage.

Sections Tₛₜ, Tₛᵦ, and Tₕ are sampled from the papillomacular bundle fibers and have the smallest RGC fibers. A low mean RNFL may be attributable to a combination of a thin RNFL and low number of RGC axons that have a significantly smaller mean neurotubule number. The sparse axons in the papillomacular bundles could be a fixation artifact if the smaller axons were not adequately preserved by the perfusion of fixative. A similar pattern of sparse axons was observed in the two eyes studied which should give some assurance that the pattern was not unique to a single eye; however, it is possible that the neurotubules in the small axons of the papillomacular bundle degenerated faster than the larger axons found elsewhere. The neurotubules polymerize and depolymerize very quickly and the smaller axons may be more fragile than the larger ones. The sparse axons and fewer nonsparse axons within octant Tₙ and angular sections Tₛₜ and Tₛᵦ could also be attributed to bilateral optic atrophy (BOA).

The packing density was greatest for sections Sₙₙ, Sₙₘ, Sₜₘ, Iₜₘ, and Iₙₘ which are sampled from arcuate fibers. The arcuate portions of the RNFL are frequently involved in glaucoma and optic neuropathies. Higher birefringence in the superior and inferior region of the eye can be attributed to a combination of higher neurotubule packing density, larger axons, and less compartmentalization by glial tissue than other regions of the eye.

Correlation between Birefringence and RGC Axon Organelles

The high correlation between Aₛ and PR/UD in both eyes is interesting because axoplasmic regions of RGC axons are the only

<table>
<thead>
<tr>
<th>Statistical Model</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson’s correlation</td>
<td>0.67</td>
<td>0.005</td>
</tr>
<tr>
<td>Multilevel model</td>
<td>0.5</td>
<td>0.04</td>
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</tbody>
</table>

TABLE 4. Correlation between PR/UD and ρₚₑₙₙ in Subject 57204
areas that neurotubules can inhabit. In addition, there was a significant correlation between $A_a$ and $\text{PR}/\text{UD}$ in subject 1853. These findings suggest that a structure within the axoplasm is the source of the birefringence signal. The mean ($u$) (neurotubule packing density) was more significantly correlated with $\text{PR}/\text{UD}$ than the mean $A_a$ for subject 57204. This suggests that the birefringence surrounding the ONH results from a difference in

![Figure 9](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933244/)

**Figure 9.** Average birefringence measurements calculated from EPS-OCT $\text{PR}/\text{UD}$ radial area and ring scans plotted with respective regional $\rho_{\text{RNFL}}$ for subjects (A) 57204 (B) and 1853. Area and ring scans of $\text{PR}/\text{UD}$ measured within angular regions that were sampled for neurotubule density surrounding the ONH were averaged within the respective angular section region, resulting in an equal number of $\text{PR}/\text{UD}$ measurements for every $\rho_{\text{RNFL}}$. Value. N, nasal; T, temporal; S, superior; I, inferior.

![Figure 10](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933244/)

**Figure 10.** $\text{PR}/\text{UD}$ (proportional to birefringence) measurements from radial area and ring scans in subject 57204 were averaged within its respective angular section around the (ONH) and superimposed on (A) mean ($A_a$) modes for subject 57204 and (B) mean ($A_a$) and ($A_x$) modes for subject 1853 measured in corresponding regions of the same eye. N, nasal; T, temporal; S, superior; I, inferior.

![Table 5](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933244/)

**Table 5.** RGC Morphologic measurements Correlated with Phase Retardation Per Unit Depth ($\text{PR}/\text{UD}$, Proportional to Birefringence) for Subjects 57205 and 1853

<table>
<thead>
<tr>
<th>RGC Axon Morphologic Measurement</th>
<th>Abbreviation</th>
<th>57204 OD</th>
<th>1853 OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Least-squares linear slope (number of NTs per unit $A_x$ area), NT/µm²</td>
<td>Mean $u$</td>
<td>0.70*</td>
<td>0.68</td>
</tr>
<tr>
<td>Mean RGC axoplasmic area mode, µm²</td>
<td>Mean $A_x$</td>
<td>0.50*</td>
<td>0.81*</td>
</tr>
<tr>
<td>Mean axon area mode, µm²</td>
<td>Mean $A_a$</td>
<td>0.47</td>
<td>0.0600</td>
</tr>
<tr>
<td>Neurotubule density (number of NTs per unit RNFL area), NT/µm²</td>
<td>Mean $\rho_{\text{RNFL}}$</td>
<td>0.67*</td>
<td>0.61</td>
</tr>
<tr>
<td>Axoplasmic fractional area (per unit RNFL bundle area), %</td>
<td>Mean $f$</td>
<td>0.34</td>
<td>0.51</td>
</tr>
<tr>
<td>Mitochondrial fractional area (per unit RNFL bundle area), %</td>
<td>Mean $x_m$</td>
<td>0.02</td>
<td>0.9500</td>
</tr>
<tr>
<td>Mitochondria-containing axon profile fraction (per unit RNFL bundle area), %</td>
<td>Mean $m_p$</td>
<td>—</td>
<td>0.24</td>
</tr>
<tr>
<td>Axonal membrane length (per unit RNFL bundle area), µm⁻¹</td>
<td>Mean $L_{\text{m}/A_b}$</td>
<td>0.24</td>
<td>0.5670</td>
</tr>
</tbody>
</table>

RGC axoplasmic area ($A_x$) mode, neurotubule (NT) number ($k$) versus $A_x$ least-squares linear slope ($u$), and fractional area of axoplasm per unit nerve fiber bundle ($f$) measurements are associated with nonsparse axons only. Axon area ($A_a$) mode, mitochondrial fractional area ($x_m$), mitochondria-containing axon profile fraction ($m_p$), and length of axonal membrane per unit nerve fiber bundle area ($L_{\text{m}/A_b}$) measurements are associated with both sparse and nonsparse axons.

* Morphologic parameters that had a significant correlation with $\text{PR}/\text{UD}$. 

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REFERENCES


