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Three-Dimensional Histomorphometry of the Normal and Early Glaucomatous Monkey Optic Nerve Head: Neural Canal and Subarachnoid Space Architecture

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Abstract

Purpose—To delineate three dimensionally the neural canal landmarks—Bruch's membrane opening (BMO), anterior sclera canal opening (ASCO), anterior laminar insertion (ALI), posterior laminar insertion (PLI), and posterior scleral canal opening (PSCO)—and the anterior-most aspect of the subarachnoid space (ASAS), within digital three-dimensional (3-D) reconstructions of the monkey optic nerve head (ONH).

Methods—The trephinated ONH and peripapillary sclera from both eyes of three early glaucoma (EG) monkeys (one eye normal, one eye with laser-induced EG) were serial sectioned at 3- μ m thickness, with the embedded tissue block face stained and imaged after each cut. The images were aligned and stacked in a 3-D volume, within which the BMO, ASCO, ALI, PLI, PSCO, and ASAS were delineated in 40 digital, radial, and sagittal sections. An ellipse was fitted to the 80 BMO points to establish a BMO zero reference plane, on which all other points were projected. The distance from each projected point to the BMO centroid (offset) and BMO zero reference plane (depth) were calculated and compared regionally between normal and EG eyes, both overall and within each monkey, by analysis of variance.

Results—BMO was the clinically visible optic disc margin in all six eyes. The neural canal architecture was highly variable in the three normal eyes. Radial expansion of the neural canal was greatest posteriorly in the EG eyes. Axial elongation of the canal was less pronounced overall but was regionally present within all three EG eyes. ASAS was regionally radially expanded and anteriorly displaced within two of the three EG eyes.

Conclusions—Profound deformation of the neural canal and ASAS architecture are present in young adult monkey eyes at the onset of ONH surface change in early experimental glaucoma.

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We have proposed that intraocular pressure (IOP)–related optic nerve head (ONH) connective tissue damage is a defining feature of glaucomatous cupping.^{1,2} By this, we mean that for an individual ONH to demonstrate true glaucomatous cupping (defined to include posterior deformation of the lamina and excavation of the peripapillary sclera), its connective tissues must become damaged and undergo mechanical yield and/or remodeling in a process that is governed by the distribution of IOP-related stress and strain, regardless of the mechanism of insult or the level of IOP at which the insult occurs.^{3,4}

We have previously demonstrated permanent deformation of the ONH connective tissues within serial histologic sections² and two-dimensionally delineated, digital, three-dimensional (3-D) reconstructions of monkey ONHs with early experimental glaucoma.² In both the histologic and 3-D reconstruction studies, expansion of the anterior portion of the neural canal (Bruch's membrane opening [BMO] and the anterior laminar insertion) was present within the optic nerve heads of the monkey with early glaucoma (EG).

In the present report, we introduce a new method for 3-D delineation of ONH and peripapillary scleral landmarks (see Fig. 1) within the previously reported ONH connective tissue reconstructions² and test the method's reproducibility. In this, the first of five reports devoted to quantification of ONH and peripapillary scleral neural and connective tissue architecture, we concentrate on the architecture of the neural canal wall and its relationship to the anterior-most aspect of the subarachnoid space.

We have proposed the term *neural canal* for the axonal pathway through the eye wall,² which includes a prescleral region as well as the traditional scleral canal. The neural canal extends from its clinically visible internal opening (BMO, clinically referred to as the disc margin) to its clinically invisible external opening (the posterior scleral canal opening; see Figs. 1C, 2).

Defining the neural canal in this manner is clinically significant, because the anterior-most portion of the canal (the BMO) is easily identified in clinical and histologic images. BMO thus provides a common and relatively stable zero reference plane for both clinical and histologic measurements. In addition, histologically detectable alterations in the shape and size of the neural canal occur early in the glaucomatous optic neuropathies, and these alterations may soon become clinically detectable by imaging modalities developed to capture the deep connective tissue architecture of the disc.⁵⁻¹³

Deformation of the connective tissues of the neural canal in early glaucoma is important because it is evidence that the tissues which underlie the clinical phenomenon of glaucomatous excavation are damaged early in the neuropathy. Three dimensionally quantifying these deformations also allows us to construct and refine computational finite element models of IOP-related connective tissue stress and strain within these same ONHs.¹⁴⁻¹⁷

In this article, we report the first clinical visualization of the histologically derived neural canal landmarks—BMO, the anterior scleral canal opening (ASCO), the anterior laminar insertion point (ALI), the posterior laminar insertion point (PLI), the posterior scleral canal opening (PSCO), and the anterior-most projection of the subarachnoid space (ASAS)—by digitally superimposing them onto confocal scanning laser tomographic (CSLT) images. Using these landmarks, we detected and characterized ONH neural canal connective tissue deformation at a very early stage of the disease.

Materials and Methods

Animals

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Three male cynomolgus monkeys, approximately 8 years of age, were used (Table 1 of our previous report²).

ONH Surface Compliance Testing and Early Glaucoma

We have described our confocal scanning laser tomography (CSLT)-based ONH surface compliance testing strategy.^{1,18} Briefly, compliance testing involved measurement of the mean position of the disc (MPD), which describes the position of the surface of the ONH relative to the peripapillary retinal nerve fiber layer. Both eyes of each monkey were compliance tested on three separate occasions to provide the prelaser $MPD_{Baseline} \pm 95\%$ confidence interval (CI) for normal eyes. Then, experimental IOP-elevation was laser induced in one eye of each monkey, and compliance testing of both eyes was repeated at 2-week intervals until the onset of permanent posterior deformation of the ONH surface (defined as $MPD_{Baseline} \pm 95\% CI < \text{postlaser } MPD_{Baseline} \pm 95\% CI$) in the lasered eye (the early glaucoma, or EG eye) on two successive postlaser imaging sessions, at which point the monkey was killed (see Fig. 1 in our previous publication² for details).

Monkey Euthanatization and Perfusion Fixation at Prescribed IOP

For each monkey, both eyes were cannulated with a 27-gauge needle under deep pentobarbital anesthesia, and the IOP was set to 10 mm Hg with an adjustable saline reservoir. After a minimum of 30 minutes, the monkey was perfusion fixed via the descending aorta with 1 L of 4% buffered hypertonic paraformaldehyde solution followed by 6 L of 5% buffered hypertonic glutaraldehyde solution. Both the paraformaldehyde and glutaraldehyde were buffered in 1 M Sorenson's solution (0.15 M KH_2PO_4 and 0.85 M Na_2HPO_4). Osmolarity of each solution was approximately 0.4 osm/L. After perfusion fixation, IOP was maintained for 1 hour, after which each eye was enucleated, all extraorbital tissues were removed, and the anterior chamber was removed 2 to 3 mm posterior to the limbus. By gross inspection, perfusion was excellent in all six eyes. The posterior scleral shells with intact ONH, choroid, and retina were placed in 5% glutaraldehyde solution for storage.

Generation of the Aligned Serial Section Images of Each Optic Nerve Head

In each eye, the ONH and surrounding peripapillary sclera were trephined (6-mm-diameter), pierced with four to seven 10-0 polypropylene alignment sutures (Prolene; Ethicon, Somerville, NJ), and photographed to document the position of the alignment sutures in the clinical ONH surface. The tissue was dehydrated through a series of graded ethanol baths, cleared in chloroform, and infiltrated with paraffin (TissuePrep 2; Fisher Scientific, Pittsburgh, PA) with an automatic tissue processor (Tissue-Tek II; Ames Division, Miles Laboratories, Inc., Elkhart, IN). The specimen was oriented so that superior was up, embedded in paraffin, and mounted on a microtome (RM2165; Leica, Wetzlar, Germany) so that the vitreous side of the ONH surface was visible in the block in a clinically appropriate orientation (see Fig. 2 of our previous publication²). A 1080×1520 -pixel charge-coupled device [CCD] camera (Spot RT; Diagnostic Instruments, Sterling Heights, MI) was positioned so that an image of the centered, embedded tissue block surface could be acquired at a resolution of $2.5 \times 2.5 \mu\text{m}$ per pixel (see Fig. 3 of our previous publication²). The block was then sectioned at $3.0\text{-}\mu\text{m}$ thickness without imaging until the ends of at least two fiducial sutures were visible in the image. At that point, a 1:1 (vol/vol) mixture of ponceau S and acid fuchsin stains was manually applied to the surface of the embedded tissue block, staining only the exposed face of the connective tissues. The excess stain was then blotted away, and the stained surface of the embedded tissue block was

imaged. Then, another 3.0- μm section was cut away, and the staining and imaging process was repeated.

Thus, for each ONH, imaging began as close to the vitreoretinal interface as possible and continued through the lamina cribrosa and approximately 200 μm into the retrolaminar orbital optic nerve. Depending on the degree of tilt of the embedded tissue, the number of sections obtained per eye ranged from 222 to 411. The serial section images were aligned in the z -direction (anterior to posterior) by registering the cut ends of the embedded polypropylene sutures that were visible within each section with custom software (see Fig. 4 of our previous publication²).

Generating Colorized, Stacked-Section, 3-D ONH Reconstructions

For each ONH, the aligned serial section images were stacked at 3.0- μm intervals (the section thickness and therefore the distance between each image) into a 3-D reconstruction of the ONH and peripapillary scleral connective tissues consisting of approximately $1080 \times 1520 \times 411$ voxels, each $2.5 \times 2.5 \times 3.0 \mu\text{m}$. A 3-D ONH reconstruction can occupy up to 2.25 GB of memory, and so it was necessary to reduce its memory footprint to facilitate responsive interaction with the digital data. Hence, custom software was used to reduce the color map of each reconstruction from 16 million to 256 colors while maintaining full volumetric resolution, thereby allowing the entire 3-D reconstruction to be loaded into memory and manipulated on a Windows-based, graphics workstation.

3-D Delineation of ONH and Peripapillary Scleral Landmark Points within 40 Digital, Radial, Sagittal Slices of Each Reconstruction

Using custom software (based on the Visualization Toolkit [VTK] Clifton Park, NY), the 3-D ONH reconstruction was loaded into memory on a remote Linux server with four, 64-bit, Itanium2 processors, and 32 GB RAM. The delineator assigned the approximate center of the neural canal as that reconstruction's center of rotation, around which 40, 7-voxel-thick, radial, sagittal slices of the digital 3-D reconstruction are served at 4.5° intervals to the delineator's workstation (Fig. 1) across a Gigabit Ethernet network.

Within each 7-voxel-thick, radial, sagittal data slice, the delineator marked seven landmark surfaces (Fig. 1E, 1F), and six pairs of neural canal landmark points (one point on each side of the canal; Fig. 1E, 1G). The landmark surfaces were (1) the internal limiting membrane (vitreoretinal interface); (2) the anterior and posterior surfaces of the peripapillary sclera; (3) the anterior and posterior surfaces of the lamina cribrosa; (4) the external surfaces of the central retinal vessels; (5) Bruch's membrane; (6) the neural canal wall (the boundary between the neural and connective tissues beginning anteriorly at Bruch's membrane opening, proceeding posteriorly along the border tissues of Elschnig, through the scleral canal, and along the pia mater boundary to the posterior edge of the reconstruction); and (7) the subarachnoid space (internal surface of the dural sheath and external surface of the pia mater).

The neural canal landmark points (Fig. 1G) are (1) Bruch's membrane opening (BMO); (2) the anterior scleral canal opening (ASCO); (3) the anterior laminar insertion (ALI); (4) the posterior laminar insertion (PLI); (5) the posterior scleral canal opening (PSCO); and (6) the anterior-most aspect of the subarachnoid space (ASAS).

Within each 7-voxel-thick, radial, sagittal data slice, the seven surfaces or boundaries and the six neural canal landmarks are marked within the central 1-voxel-thick sagittal section image by mouse-clicking, while the cross hairs were over the desired landmark location (Fig. 1G). While marking in the sagittal section view, the delineator simultaneously viewed an adjacent window showing the cross hairs' 3-D location within a digital transverse section image slaved

to the sagittal data slice view (Fig. 1H). Thus, within the 3-D reconstruction, each landmark or surface point was chosen with full awareness of the point's location within both the sagittal and transverse planes. If the landmark or structure was not readily visible in the central 1-voxel-thick sagittal section image, the delineator scrolled through the adjacent six 1-voxel-thick sagittal section images (three 1-voxel-thick sections in front of the central section, and three behind) to locate a section in which the landmark could be clearly identified and marked. The 3-D Cartesian coordinates for each mark were saved and were edited later by either deletion or adjustment of location.

Delineation of the 13 landmark surfaces, boundaries, and points within the 40 radial, digital, sagittal slices of each 3-D ONH reconstruction generated a 3-D point cloud that included each of the marked structures (Fig. 1F). Each point cloud contained the precise mathematical location of an anatomic surface (e.g., the anterior surface of the sclera), an opening in the neural canal (e.g., BMO), or concentric feature in the ONH or peripapillary sclera (e.g., ASAS). Entire mark sets could be visualized and edited by individual surface, boundary, or neural canal landmark identifiers, either during initial marking or on later inspection.

Clinical Alignment and Visualization of Neural Canal Landmark and Overlay Maps

For this study, only data from the six neural canal landmark points were assessed (Figs. 1E, 1J, 2). To establish the clinical orientation of each 3-D ONH reconstruction, a high-resolution 3-D reconstruction of the central retinal vessels and the neural canal landmark points was constructed and three dimensionally overlaid onto a clinical fundus photograph or CSLT image (Fig. 2). By adjusting the 3-D orientation of the vessel-marks complex, the two were best matched to the clinical image, establishing the rotation angle necessary to achieve true anatomic orientation of the 3-D marks.

Neural canal landmarks for each eye of each monkey were visualized relative to the central retinal vessels and a clinical image, as seen in Figure 2. Neural canal landmark overlay maps for each monkey were created by digitally converting the left eye data to right eye configuration and overlaying the left eye onto the right eye data for comparison (Fig. 2, right).

BMO Zero Reference Plane and Neural Canal Landmark Offset and Depth Quantification

For each 3-D ONH reconstruction, a least-squares ellipse was fitted to the 80 marks defining BMO, creating a BMO zero reference plane (Fig. 1K, 1L). The centroid of the BMO ellipse established the center point for all measurements. All subsequent measurements were made relative to the BMO zero reference plane, the BMO centroid, and the normal vector to the BMO zero reference plane at the BMO centroid (BMO normal vector).

For each landmark, its concentric ring of 80 landmark points (2 for each of the 40 radial sagittal slices) was projected onto the BMO zero reference plane along the BMO normal vector. Two parameters were defined for each landmark point and structure (Fig. 1K): (1) *depth*: the anterior-to-posterior distance of each marked point to the BMO zero reference plane; and (2) *offset*: the distance (within the BMO zero reference plane) of each projected mark from the BMO centroid.

Regionalization

Offset and depth data for each neural canal landmark were pooled for eight anatomic regions, superior (S), superonasal (SN), nasal (N), inferonasal (IN), inferior (I), inferotemporal (IT), temporal (T), and superotemporal (ST), according to the previously established clinical orientation (Fig. 2). The S, N, I, and T regions contained all marks within 60° sections of the ONH centered about the S-I and N-T clinical axes, and the SN, IN, IT, and ST regions contained all marks in 30° radial sections of the ONH centered about the SN-IT and IN-ST axes.

Interdelineator Variability and Intradelineator Reproducibility

Interdelineator variability was assessed by having five delineators distinguish the landmark points of both eyes of all the three monkeys. Intradelineator reproducibility was assessed by having two of the five delineators distinguish all landmarks of both eyes of monkey 3 two additional times at least 2 weeks apart. The effect of delineator (overall and within each monkey) and delineation day (for two delineators on monkey 3 only), were assessed within separate factorial analyses of variance (ANOVA).

Statistical Analysis

A factorial ANOVA was used to assess the effects of delineator, region, and treatment group (normal or EG) on the parameters *offset of BMO*, and *offset and depth of ASCO, ALI, PLI, PSCO*, and *ASAS*, both overall and between the two eyes of each monkey.

Results

Descriptive data for the three monkeys and six eyes are reported in Table 1 of our previous publication.²

Experimental Glaucoma

Monkeys 2 and 3 were killed three weeks and monkey 1 six weeks after the onset of CSLT detection of ONH surface change (permanent posterior deformation of the ONH surface). In monkeys 1 and 2, IOP elevations were moderate, with only one measurement higher than 30 mm Hg. In monkey 3, elevated IOP was not detected (Table 1, Fig. 1 of our previous publication²), but there was clear onset of experimental glaucoma as determined by CSLT-based ONH surface compliance testing.

Qualitative Size and Shape of BMO and the Neural Canal

The size and shape of the neural canal of both the normal and EG eyes of each monkey are represented in Figure 2. In each eye, BMO overlaid the clinically visible optic disc margin. While the two eyes of each monkey demonstrated marked similarity in size, shape, enlargement (the degree to which the PSCO was larger than BMO) and obliqueness (the degree to which the PSCO was off-center relative to BMO), there were clear qualitative differences in neural canal architecture between the three animals (Fig. 2), with monkeys 1 and 3 demonstrating substantial enlargement and obliqueness relative to monkey 2.

Neural canal landmark overlay maps demonstrated the relative change in size and shape of each component of the neural canal architecture in the EG eye (solid lines) compared to the contralateral normal control eye (dotted lines) of each monkey (Fig. 2). Whereas profound expansion of the posterior neural canal landmarks was regionally apparent in the EG eyes of monkeys 1 and 3, only minimal changes were present within the EG eye of monkey 2.

Overall and Intramonkey Treatment Effects on Each Parameter: ANOVA Results

Overall Offset—Table 1 shows the change in offset (expansion within the plane of BMO) of the pooled EG eyes (Δ EG) compared with the pooled normal eyes for all five delineators. All five delineators detected similar overall expansions in all the neural canal structures due to early glaucoma, with the more posterior structures (PLI, PSCO, and ASAS) showing statistically significant early glaucomatous expansion ($P < 0.05$). Note that since the structural offset is measured radially from the centroid of BMO, the values in Table 1 are indicative of only half of the total change in structural diameter.

Overall Offset within Monkeys—Offset overlay maps are presented for each monkey in Figure 2. Statistically significant differences in overall offset for all three monkeys are reported in Table 2 for delineator 1 (JCD). Monkey 1 demonstrated statistically significant expansion of all five neural canal structures in the EG eye, whereas monkeys 2 and 3 showed significant expansion in the three posterior-most structures only ($P < 0.05$). Several neural canal landmarks in monkey 1 approach an average diameter increase of 10% due to EG.

Overall Regional Offset—Overall offset by region was assessed for each parameter by ANOVA; however, no delineators observed statistically significant treatment effects in regional offset for the pooled normal and EG eyes (data not shown). This finding suggests that while regional change was present within each EG eye, the pattern among the three EG eyes was not consistent enough to achieve statistical significance of all eyes pooled by treatment.

Regional Offset by Monkey—Statistically significant regional offset for each individual monkey is presented in Figure 3, left, for delineator 1 (similar results were obtained for the other four delineators). Although all three animals demonstrated significant regional expansion of the neural canal in the EG eye, there was no consistent pattern. Significant regional changes seemed to occur in the more posterior structures (PLI, PSCO, and ASAS) as is reflected in the overall results (Table 1).

Overall Depth—Table 3 shows the change in depth (anterior-to-posterior canal elongation perpendicular to the plane of BMO) of the pooled EG eyes compared with the pooled normal eyes for all five delineators. Although there are small differences, all five delineators detected a similar pattern of posterior elongation of the posterior neural canal landmarks within the EG eyes that are smaller than the offset changes reported in Table 1. Small posterior or anterior changes in the anterior subarachnoid space were present but achieved statistical significance for only two of the five delineators.

Overall Depth by Monkey—Statistically significant changes in depth for all three monkeys are reported in Table 4 for delineator 1 (similar results were obtained for the other four delineators). Monkeys 2 and 3 demonstrated significant posterior displacement of the ALI and PLI within the EG eye, which may be an early manifestation of glaucomatous excavation. Monkeys 1 and 3 demonstrate a significant anteriorization of the ASAS, which may be an early manifestation of damage and/or thinning of the peripapillary sclera. In general, the magnitudes of change in depth (Table 4) are small than the magnitudes of change in offset (Table 2).

Overall Regional Depth—Overall depth by region was assessed for each parameter by ANOVA; however, few delineators showed statistically significant treatment effects in regional depth for the pooled normal and EG eyes (data not shown). This finding suggests that, although regional depth changes were present within each EG eye (Fig. 3), the pattern among the three EG eyes was not consistent enough to achieve statistical significance.

Overall Regional Depth by Monkey—Statistically significant regional depth for each monkey is presented in Figure 3, right, for delineator 1 (similar results were obtained for the other four delineators). Although all three animals demonstrated significant regional expansion of the neural canal in the EG eye, there was no consistent pattern. Regional depth changes occurred in the anterior and posterior laminar insertion points (ALI, PLI) of two of the three EG eyes and all three EG eyes demonstrated anteriorization of some portion of the ASAS.

Interdelineator Variability

The effect of delineator for both offset and depth data were analyzed overall and for each monkey by ANOVA. Although the effect of delineator within the overall data was not

significant for offset ($P = 0.077$), it was for depth ($P < 0.001$). The delineator was significant ($P < 0.001$) for both depth and offset in all three monkeys when considered individually; however, their effect compared with the treatment effect was small for offset and modest for depth. Tables 1 and 3 show the overall differences in offset and depth, respectively, for each landmark for all five delineators. Generally, offsets and depths were similar among delineators (Tables 1, 3). Despite statistically significant interdelineator variability, all delineators consistently detected overall glaucomatous expansion of the neural canal (Table 1). The delineators were less consistent in detecting the glaucomatous posteriorization of the neural canal landmarks (Table 3) and anteriorization of the ASAS (Table 3) because these changes were of smaller magnitude.

Intradelineator Repeatability

The reproducibility of two delineators marking both eyes of monkey 3 on three different days at least 2 weeks apart was assessed by ANOVA and is presented for both offset and depth in Tables 5 and 6, respectively. The effects of delineator and delineation day were statistically significant for both depth and offset (ANOVA, $P < 0.01$). However, the data in Tables 5 and 6 demonstrate that the offset and depth differences attributable to delineator and day are smaller than the differences due to treatment, and both the normal eye data and the treatment effects are remarkably repeatable within the three delineation days and between the two delineators.

Discussion

Accurately characterizing ONH and peripapillary scleral connective tissue architecture has been the goal of a large and important body of literature,^{1,5,19-60} which was extensively reviewed in our previous report.² In that article, we introduced our method for high-resolution, digital, 3-D reconstruction of the ONH tissues and used a 2-D delineation strategy to visualize and measure differences within the connective tissue geometries of both eyes of three monkeys with early experimental glaucoma in one eye.²

In this report, we introduce a new method for 3-D delineation and quantification of ONH and peripapillary scleral architecture within color-mapped 3-D ONH reconstructions from both eyes of the three monkeys with experimental glaucoma in one eye. We used the 3-D–delineated marks to characterize quantitatively the neural canal architecture and its relationship to the anterior-most aspect of the subarachnoid space and to assess rigorously both the interdelineator variability and repeatability of the method.

Our method of 3-D reconstruction, delineation, and quantification of ONH and peripapillary scleral landmark points is reproducible for the neural canal landmarks reported herein. Generally, the absolute offset and depth values as well as the magnitude of the EG treatment effects (both overall and for each monkey) were similar among delineators. Two delineators consistently detected similar levels of difference in the EG versus normal eye of one monkey within delineations made on three different days. However, both interdelineator variability and intradelineator reproducibility was better for offset than depth. Two factors may contribute to this difference. First, the canal depth is much smaller than its radius, and so small differences in landmark point locations have bigger impact in the depth measures. Second, treatment differences in offset were larger than in depth, the implications of which are now discussed.

The principal findings of this report are as follows. First, the clinical alignment of each reconstruction according to the vessels and neural canal architectural landmarks suggests that BMO was the clinically visible optic disc margin in these six eyes. This finding is contrary to the existing literature^{22,61-63} and may pertain only to these animals. We will confirm these findings in a larger group of monkey eyes in a future report.

In their classic text, Hogan et al.⁶² suggested that, although Bruch's membrane can overhang the anterior scleral canal opening, it is not usually visible alone or when covered with retinal pigment epithelium (RPE). They reported that the scleral lip (border tissues of Elschnig^{22, 64}; Fig. 1) is the clinically visible optic disc margin in human eyes and visible portions of the scleral lip and sclera covered by either RPE or choroid underlie nonpigmented (scleral) and pigmented (RPE or choroidal) peripapillary crescents. Fantes and Anderson⁶¹ reached similar conclusions in a group of 12 human cadaveric eyes with intraocular tumors that were photographed before enucleation and then studied histologically.

In our young adult monkey eyes, the connective tissues of the choroidal border zone (the border tissues of Elschnig^{22, 64}) are robust and rise up to fuse with Bruch's membrane, which continues as a cantilevered connective tissue flange of variable length to its end (BMO; Figs. 1-4). Figure 4 unequivocally demonstrates that when our 3-D reconstructions are aligned with high-resolution optic disc photographs (only available for monkey 3), BMO is the clinically visible optic disc margin. While only definitive for this eye, the implications of this finding are important. In most monkey eyes, BMO is both clearly present clinically and easily delineated within histologic reconstructions. If it can also be detected within high-resolution, spectral-domain optical coherence tomography (OCT) scans,^{7, 9, 12, 65, 66} then a BMO-based, zero-reference plane for clinical imaging of the ONH may be possible. More extensive histologic and 3-D histomorphometric evidence of the relationship between BMO and the clinically visible optic disc margin is being generated in a large group of normal monkey eyes and will be the subject of a future report.

Second, BMO, the internal entrance to the neural canal,² does not necessarily reflect the size, shape, and relative position of the external neural canal (PSCO), and the degree to which this is true is highly variable among normal monkey eyes (Figs. 2-5). Two features of the neural canal separately contribute to the architecture of the canal wall, which may influence ONH susceptibility⁴: (1) neural canal enlargement (the degree to which the optic nerve increases in size within the neural canal), defined as the difference in size between the external (PSCO) and internal (BMO) neural canal opening; and (2) canal obliqueness (the angle and direction of the canal's passage through the sclera), which can be defined by the transverse shift of the external relative to the internal neural canal opening.

It should be noted that in making these distinctions, we have adopted the term *neural canal enlargement* to describe canal architecture that exists as a result of embryologic development (before intervention). We use the term *neural canal expansion* to describe experimentally induced radial expansion of the canal beyond its preexisting state.

The neural canal area enlarged 163%, 53%, and 100% in the normal eyes of monkeys 1, 2, and 3, respectively, within the data from delineator 1 (Table 7), and these values are similar for all five delineators.

Neural canal obliqueness is calculated by connecting the BMO centroid to the PSCO centroid and measuring the angle between this vector and the vector normal to the BMO zero reference plane (Fig. 5). In the case of a perfectly straight-walled cylindrical canal, the centroids of BMO and the PSCO align, and canal obliqueness is zero. As the PSCO-centroid shifts laterally away from the normal vector to the BMO zero reference plane at the BMO-centroid, canal obliqueness increases. Neural canal obliqueness varies from 9° to 70° for the three normal eyes within the data from delineator 1 (Fig. 5, Table 7) and these values are similar for all five delineators. Canal obliqueness is regional, and greatest on the nasal side of the ONH in four of the six eyes, with monkey 2 showing relatively little neural canal obliqueness.

Neural canal obliqueness may affect ONH susceptibility in two ways: First, the greater the obliqueness of the canal, the more severe a turn the retinal ganglion cell axons must make to

enter the neural canal from the peripapillary nerve fiber layer (Fig. 5).²⁹ If canal obliqueness is zero, then the axons must only turn 90° on their path from the retina through the neural canal and into the orbital optic nerve. However, when canal obliqueness is substantial, as is the case in monkeys 1 and 3, the axonal path around BMO for a subset of axons is much more tortuous, which has been shown to influence axonal transport.²⁹

Second, the obliqueness of the canal wall determines the shape of the scleral flange which is a connective tissue cantilever of highly variable geometry extending from the PSCO to BMO consisting of the border tissues of Elschnig (between BMO and ASCO) and a scleral (between ASCO and PSCO) component (Fig. 5). The peripapillary sclera has been shown to be the site of substantial stress and strain concentration within initial finite element models of the human^{15,17} and monkey ONH.¹⁴ These tissues are also the path by which the communicating branches of the posterior ciliary arteries achieve the lamina cribrosa beams, the circle of Zinn-Haller and the immediate retrolaminar optic nerve.⁶⁷ The contributions of variation in neural canal architecture within normal monkey eyes to their susceptibility to glaucomatous damage remain to be determined.

Permanent changes in neural canal and subarachnoid space architecture are present at the earliest detectable stage of experimental glaucoma in the young adult monkey eye. We believe these changes are permanent and irreversible because (1) each animal had IOP lowered to 10 mm Hg in both eyes for at least 30 minutes before perfusion fixation; and (2) pressure elevations in the glaucomatous eye of the two monkeys on which it was checked were not that high immediately before anterior chamber manometer insertion (37 mm Hg in monkey 2 and 18 mm Hg in monkey 3). Although the magnitude of posterior change we report may have been less had we allowed more time for the tissues to equilibrate at an IOP of 10 mm Hg before perfusion, in a previous study, only minimal levels of anterior ONH surface movement were detected 45 minutes after IOP lowering in early glaucoma eyes.⁶⁸ We believe that permanent change similar to the magnitude we report would be present in these eyes, regardless of equilibration time.

All neural canal landmarks demonstrated at least regional radial expansion in the EG eyes, which was greatest within the external portions of the canal (the PLI and PSCO). However, the size of Bruch's membrane opening, which is used by all imaging devices for both longitudinal image registration and for parameter generation, was also significantly increased in the EG eye of one monkey. Although there was significant glaucomatous expansion of the canal within each monkey (Fig. 3), the regional pattern of radial expansion was not consistent in all 3 EG eyes.

All neural canal landmarks demonstrated posterior migration relative to BMO (axial elongation of the canal), with significant displacement of PLI overall and both PLI and ALI in two of three monkeys. The PSCO was significantly posteriorly displaced in the EG eye of one monkey. Regional glaucomatous canal elongation was present within each monkey (Fig. 3), but again with no pattern consistent in all three EG eyes.

Finally, the anterior subarachnoid space expanded radially in the EG eyes both overall and in all three monkeys. However, rather than demonstrating posterior migration, the subarachnoid space moved anteriorly both overall and in two of three EG eyes. Regional anteriorization of the subarachnoid space was present within the same two monkeys that demonstrated focal regional expansion (Fig. 3).

This anteriorization and/or expansion of the anterior-most aspect of the subarachnoid space may represent peripapillary scleral thinning immediately adjacent to the canal, which suggests that peripapillary scleral damage may be present very early in the neuropathy. Quantitative

overlay maps of peripapillary scleral thickness and position in these same three monkeys will be the subject of a future report.

Radial expansion and axial elongation of neural canal landmarks probably represent the initial connective tissue components of clinical excavation. This excavation is composed of three connective tissue components: (1) radial expansion of the neural canal relative to BMO; (2) posteriorization of the anterior laminar insertion site (ALI) resulting from either axial elongation of the canal or physical disruption of the anterior-most laminar beams; and (3) posterior deformation of the peripheral lamina cribrosa surface (relative to BMO). In this study, we have established that two of these phenomenon are present, at least regionally, in all three EG eyes. Clinically oriented lamina cribrosa position and thickness overlay maps for each EG monkey will be included in our next report and will allow us to test the hypothesis that peripheral laminar deformation and thinning colocalizes with neural canal radial expansion in these same EG eyes.

Although several researchers have reported that glaucomatous eyes measured clinically⁶⁹⁻⁷¹ and after death^{37,72} do not have larger canal diameters, radial expansion of the neural canal in glaucoma has been reported previously in a series of reports either identified as such⁷³⁻⁷⁶ or in the larger context of glaucomatous excavation.^{28,31} While not specifically noted, enlargement of the clinically visible optic disc margin is clearly evident within the glaucomatous eye of a series of patients with unilateral glaucomatous damage.⁶³ It was also present within two separate descriptions of the rat glaucoma model.^{6,77} The importance of our current report is that it documents the presence of neural canal expansion very early in the neuropathy in young adult monkey eyes exposed to only moderate IOP elevations. The presence of glaucomatous canal wall deformation in elderly monkeys and humans, in whom the connective tissues are likely to be more robust, remains to be determined.

A predictable pattern of axonal loss underlies glaucomatous visual field loss with the early changes traditionally ascribed to the superior and inferior poles of the ONH.^{28,35} It should be noted that in these three EG eyes, early neural canal connective tissue deformations are not consistently localized to the superior and inferior quadrants in the EG eyes of monkeys 2 and 3 (Fig. 3). A definitive study colocalizing alterations in the ONH and peripapillary retinal surface, prelaminar neural tissue thickness, and lamina cribrosa position and thickness with orbital optic nerve axonal loss in these three EG eyes will be the subject of a future report.

The limitations of our method of 3-D reconstruction have been discussed² and include: (1) Anterior-to-posterior resolution is limited to 3 μm by the fact that the current stain penetrates approximately 2.5 μm into the block; (2) the stain is applied by hand to the block face with a cotton-tipped swab, and the excess is manually removed with lens paper, and thus staining variation between section images can be substantial; (3) there are tissue shrinkage effects (both from fixation and embedding) associated with this technique, but since all eyes were treated identically, comparisons between the two eyes of each monkey and within treatment groups should be valid; and (4) we have not yet characterized physiologic, intereye differences for these parameters.

In addition, although all future monkey eye reconstructions will be aligned to clinical photos, clinical alignment of the 3-D reconstruction for these three monkeys was performed with pseudocolored CSLT images, which may not provide as clear an image of the clinical optic disc margin. However, in a subsequent alignment of both eyes of monkey 3 to clinical photographs (the only animal for which they were available; Fig. 4), no substantial shift in alignment over that achieved with CSLT images was required.

Although the treatment effects reported herein are consistent overall and within each of the three monkeys, we cannot say with certainty that the differences in the EG eyes are treatment

effects that exceed physiologic differences between the two normal eyes of a normal monkey. Both ONHs of five, bilaterally normal adult monkeys (perfusion fixed with both eyes set to an IOP of 10 mm Hg by manometer) are currently awaiting 3-D reconstruction and will be the subject of a future report.

Continuum and microfinite element models of each eye, which are derived from the 3-D landmark point-clouds described earlier, are currently under construction. These models will characterize the magnitude and distribution of IOP-related stress and strain within the connective tissues of each 3-D-reconstructed ONH. This characterization will allow us to establish the relationships between IOP-related stress and strain, ONH anatomy, and EG connective tissue damage. These relationships, once elucidated, will provide valuable insight into the biomechanical factors that contribute to individual ONH susceptibility to glaucomatous damage.

Finally, we propose that visualization of the neural canal landmarks described in this report should become an important goal of clinical glaucoma imaging for the following reasons. First, in assessing the susceptibility of an individual ONH to a given level of IOP, the 3-D architecture of the entire canal and peripapillary sclera are likely to be more important than the clinically visible size and shape of the optic disc margin (BMO). Second, clinically detectable deformation of the neural canal wall structures (subsurface structural change) may precede early surface-detected structural and functional change. Third, peripapillary pigment atrophy⁷⁸⁻⁸⁷ may be a surface manifestation of underlying neural canal architecture in both normal and glaucomatous eyes sharing etiologic links that have not been appreciated previously.

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Figure 1.

3-D delineation of ONH and peripapillary scleral landmark points within colorized, stacked-section, 3-D ONH reconstructions. (A) Digital image of a representative central horizontal histologic section from a normal monkey eye perfusion fixed at an IOP of 10 mm Hg with the nasal side of the canal magnified in (B). (C) Histologic landmarks within (B) are labeled as follows: a, BMO; b, insertion of the border tissues of Elschnig into Bruch's membrane; c, border tissues of Elschnig; d, ASCO; e, border tissues of Jacoby, which in this region are heavily pigmented; f, PLI; g, PSCO; h, ASAS; i, BM flange, extending centrally beyond the border tissues and in this case without pigmentation and not covered by RPE; j, RPE sitting on BM. (D) Principle tissues colored as follows: yellow, BM; dark blue, border tissues of Elschnig;

green, sclera; *red hatched*, branches of posterior ciliary arteries; *light blue*, subarachnoid space. (E) Neural canal landmark points analyzed in the study. (F–J) 3-D delineation methodology. Note that generation of 3-D ONH reconstructions from aligned serial section images are explained in Figures 1 and 2 of our previous publication.² (F) A total of 40 serial digital, radial, and sagittal slices (each 7 voxels thick) are served to the delineator at 4.5° intervals. (G) A representative digital sagittal slice, showing the marks for seven landmark surfaces and six pairs of landmark points, which are 3-D-delineated using linked, simultaneous, colocalization of the sagittal slice (shown), and the transverse section image (H). (I) Representative 3-D point cloud showing all delineated points for a normal monkey ONH relative to the last section image of the reconstruction. (J) The subset of the 3-D point cloud showing the neural canal landmarks reported in this study. (K) The 80 delineated BMO points are fit to an ellipse (L) that defines the BMO zero reference plane and the BMO centroid. All other neural canal landmark points are projected onto the BMO zero reference plane (M) shown for the PSCO, only, then the distance from the BMO centroid (offset, *blue lines*) and distance from the BMO zero reference plane (depth, *green lines*) are noted for each marked point (N).

Figure 2.

Clinical overlay of neural canal landmark points in both eyes of each monkey with overlay maps and regionalization for each monkey. Neural canal landmark points projected onto the BMO zero reference plane for the normal (*left*) and EG eye (*middle*) of each monkey (both in right eye configuration). To the *right*, offset overlay maps for each monkey (*solid lines*: EG eye data; *dotted lines*: normal eye data) in right eye configuration with coincident BMO centroids. Beneath and to the *left* of each offset overlay map, the depth data (in micrometers) for the central vertical section (*left*) and central horizontal section (*bottom*) are plotted in scale for reference. S, superior; SN, superonasal; N, nasal; IN, inferonasal; I, inferior; IT, inferotemporal; T, temporal; ST, superotemporal.

Figure 3.

Regional treatment effects in offset and depth (both in micrometers) by monkey. Values are the magnitude of change in the EG eye relative to its contralateral normal control (positive is radial expansion and negative is radial contraction for offset; positive is axial elongation and negative is axial contraction or anteriorization for depth). Concentric rings represent the different neural canal landmarks from internal to external: BMO, ASCO, ALI, PLI, PSCO, ASAS, respectively. The regions that achieve statistical significance ($P < 0.05$, ANOVA) are colored by landmark (bottom right).

Figure 4.

Bruch's membrane opening is the clinically visible optic disc margin within clinical photographs of monkey 3. (A) Clinical photograph of the normal right eye of monkey 3. *White boxed area* is in (G) and (J). (B) 3-D reconstruction of the central retinal vessels and BMO (red) and ASCO points (blue) for this eye are overlaid onto the clinical photograph to achieve the best qualitative alignment of the vessel branch points. Note the alignment of BMO points with the optic disc margin in the clinical photograph. *Green line*: approximate location of the digital sagittal section shown in (C). (C) Digital sagittal section in which the operator has delineated BMO on the nasal side of the canal (red dot) by positioning the cursor in this image, while simultaneously noting its position (red dot within green cross-hairs) in the slaved

transverse section image shown in **(D)**. *White boxes* in **(C)** and **(D)** are shown in magnified view in **(E)** and **(F)**, along with an estimate of their location in the clinical photograph shown in **(G)**. Labeling in **(H)**, **(I)**, and **(J)** is as follows: a, BM extending over and beyond the border tissues of Elschnig, covered by variably mottled pigment; b, probable start of viable RPE with a greater density of pigment; c, BM points that are not clearly seen in this image but are present when the brightness and contrast are adjusted (part of the normal delineation sequence); d, BMO point (*red*), which is marked by the delineator by colocalizing the cursor in image **(E)** with the *green cross hairs* of the cursor in the slaved transverse view **(F)**; e, the junction of the border tissues of Elschnig with BM; f, the border tissues of Elschnig; g, ASCO; h, heavy pigmentation within the choroid. *White dotted lines* in **(I)** and **(J)**: the border of more densely mottled pigment on BM that is visible within the clinical photographs and serial transverse section images of the 3-D reconstruction. Taken together, the images demonstrate that in this region of this eye, lightly pigmented BM extend beyond the border tissues of Elschnig and is the clinically visible optic disc margin.

Figure 5.

Enlargement and obliqueness of the neural canal in representative digital, sagittal, and nasotemporal sections of the normal eye of each monkey. BMO (red), ASCO (yellow), ALI (dark blue), PLI (green), PSCO (purple), and ASAS (blue) landmarks delineated within the central N-T sagittal section from the normal eye of each monkey, showing the obliqueness (angle between the BMO zero reference plane normal vector and the line connecting the BMO centroid [top black dot] and PSCO centroid [bottom black dot]), and anterior-to-posterior enlargement (distance between BMO marks relative to PSCO marks) of the neural canal. Neural canal enlargement and obliqueness measures for the normal eye of each monkey are given in Table 7.

Table 1

Overall Offset of Neural Canal Landmarks by Delineator

Structure	Delineator 1		Delineator 2		Delineator 3		Delineator 4		Delineator 5	
	Normal	ΔEG	Normal	ΔEG	Normal	ΔEG	Normal	ΔEG	Normal	ΔEG
BMO	594	22	598	37	600	27	611	29	590	25
ASCO	693	31	692	14	689	21	689	31	698	48*
ALI	701	33	707	30	705	41	699	37	705	46*
PLI	818	67*	818	76*	808	67*	826	69*	814	63*
PSCO	846	48*	832	68*	832	47*	834	63*	822	59*
ASAS	953	72*	959	67*	948	66	957	56*	962	64*

Treatment values (ΔEG) are the pooled EG10 eye measure minus the pooled N10 eye measure (in micrometers). Positive values represent radial expansion, and negative values represent contraction. Neural canals are described in the Methods Section and Figure 1.

* $P < 0.05$, ANOVA.

Table 2

Offset of Neural Canal Landmarks by Monkey for Delineator 1

Structure	Monkey 1		Monkey 2		Monkey 3	
	Normal	ΔEG	Normal	ΔEG	Normal	ΔEG
BMO	579	39 *	590	2	615	21
ASCO	724	71 *	648	11	712	1
ALI	726	70 *	649	11	736	4
PLI	904	99 *	721	39 *	836	54 *
PSCO	940	85 *	743	19 *	861	30 *
ASAS	1038	98 *	831	39 *	993	74 *

Treatment values (ΔEG) are the EG10 eye measure minus the N10 eye measure for each monkey and are expressed in micrometers. Positive values represent radial expansion and negative values represent contraction. Neural canal landmarks are defined and described in Methods and Figure 1.

* $P < 0.05$, ANOVA.

Table 3

Overall Depth of Neural Canal Landmarks by Delineator

Structure	Delineator 1		Delineator 2		Delineator 3		Delineator 4		Delineator 5	
	Normal	ΔEG	Normal	ΔEG	Normal	ΔEG	Normal	ΔEG	Normal	ΔEG
ASCO	35	4	33	10	39	7	37	8	34	8
ALI	41	12	48	20*	55	24*	41	10	43	12
PLI	133	16*	131	42*	135	33*	140	28*	127	14*
PSCO	146	7	135	38*	142	18*	142	25*	123	14*
ASAS	152	-17*	164	-1	162	-7	166	-10	154	18*

Treatment values (ΔEG) are the pooled EGI0 eye measure minus the pooled N10 eye measure (in micrometers). Positive values represent axial elongation, and negative values represent axial contraction or anteriorization. Neural canal landmarks are defined and described in the Methods section and Figure 1.

* $P < 0.05$, ANOVA.

Table 4

Depth of Neural Canal Landmarks by Monkey for Delineator 1

Structure	Monkey 1		Monkey 2		Monkey 3	
	Normal	ΔEG	Normal	ΔEG	Normal	ΔEG
ASCO	22	7	29	4*	53	2*
ALI	22	7	38	18*	64	14*
PLI	86	-5	166	39*	145	14*
PSCO	104	-9	181	25*	153	7
ASAS	125	-25*	185	9	146	-36*

Treatment values (ΔEG) are the EG10 eye measure minus the N10 eye measure for each monkey (in micrometers). Positive values represent axial elongation and negative values represent axial contraction or anteriorization. Neural canal landmarks are defined and described in Methods and Figure 1.

* $P < 0.05$, ANOVA.

Table 5

Monkey 3 Offset: Intradelineator Reproducibility by Day for Two Delineators

Structure	Delineator 3						Delineator 4					
	Normal			ΔEG			Normal			ΔEG		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
BMO	624	625	630	25	24	20	644	643	647	15	20	16
ASCO	691	691	681	6	-6	3	694	705	706	8	10	12
ALI	724	719	722	33*	25	24	721	736	739	20	10	16
PLI	823	835	840	47*	38*	33*	846	844	841	36*	34*	38*
PSCO	847	844	856	22	23	11	850	857	857	33*	-7	-17
ASAS	975	983	990	77*	77*	71*	979	989	999	49*	83*	69*

Treatment values (ΔEG) are the EG10 eye measure minus the N10 eye measure (in micrometers).

* $P < 0.05$, ANOVA.

Table 6
Monkey 3 Depth: Intradelineator Reproducibility by Day for Two Delineators

Structure	Delineator 3						Delineator 4					
	Normal			ΔEG			Normal			ΔEG		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
ASCO	53	51	44	0	3	8	53	57	58	2	4	1
ALI	72	73	78	18*	18*	16*	65	66	69	6	9	17*
PLI	140	153	159	16*	9	6	150	148	147	11	17*	16*
PSCO	147	150	158	2	2	-4	151	150	150	8	-3	-9
ASAS	160	168	169	-43*	-46*	-46*	165	159	161	-33*	-35*	-38*

Treatment values (ΔEG) are the EG10 eye measure minus the N10 eye measure.

* $P < 0.05$, ANOVA.

Table 7**Canal Enlargement and Obliqueness**

Monkey	Structural Measure	Treatment	
		N10	EG10
Monkey 1	BMO area (mm ²)	1.06	1.21
	PSCO area (mm ²)	2.79	3.34
	Canal enlargement (%) [*]	163	177
	Canal obliqueness (°) [†]	70	68
Monkey 2	BMO area (mm ²)	1.12	1.12
	PSCO area (mm ²)	1.75	1.84
	Canal enlargement (%) [*]	56	65
	Canal obliqueness (°) [†]	9	11
Monkey 3	BMO area (mm ²)	1.21	1.29
	PSCO area (mm ²)	2.41	2.55
	Canal enlargement (%) [*]	100	98
	Canal obliqueness (°) [†]	63	55

* Anterior-to-posterior change in neural canal area.

[†] Degree of N-T lateral shift in the canal.