

Quantifying Retinal Nerve Fiber Layer Thickness Histologically: A Novel Approach to Sectioning of the Retina

Eytan Z. Blumenthal

PURPOSE. To present a technique of ocular sectioning that enables continuous histologic measurements of peripapillary retinal nerve fiber layer (RNFL) thickness in a concentric ring around the optic disc, corresponding to similar regions measured by *in vivo* imaging techniques.

METHODS. Two pig eyes and two normal human eyes were processed using the “umbrella” technique, in which peripapillary concentric ring sections were obtained, at increasing diameters, all centered on the optic disc. Each histologic ring section contains a continuous circumferential 360° retinal slice, oriented approximately perpendicular to the retinal surface. Every histologic slice contains each axon of the retina, sectioned perpendicular to each axon’s long axis and at an equal set distance from the disc margin.

RESULTS. Ring sections from pig and human eyes are presented and correlated to known RNFL anatomy. For the two human eyes, peripapillary RNFL thickness was quantified and plotted, resulting in the expected double-hump pattern.

CONCLUSIONS. The umbrella technique provides, on a single histologic section, all necessary information for quantifying the entire RNFL layer of that eye, in a standardized fashion. This technique can simplify the process of analyzing the RNFL thickness histologically, assist in obtaining a normative database of RNFL thickness in humans, and be implemented as a histologic end point in animal studies evaluating new treatment modalities for glaucoma. (*Invest Ophthalmol Vis Sci.* 2004;45:1404–1409) DOI:10.1167/iov.03-0744

The retinal nerve fiber layer (RNFL) is made up of retinal ganglion cell axons converging to form the optic nerve. This layer is damaged in glaucoma, resulting in atrophy and thinning. In the clinical setting, the RNFL can be assessed qualitatively during ophthalmoscopy and through RNFL-enhanced photography, as well as quantitatively, using several imaging devices designed for glaucoma diagnosis and follow-up.^{1,2}

Although much has been published on imaging of the RNFL, little is known about true measurements of RNFL thickness. Of the six studies published to date on histologic quantification of RNFL thickness in primate eyes^{3–6} and human eyes,^{7,8} no two studies have approached the issue of sectioning (and processing) the retina in the same way.

Morgan et al.⁶ and Dichtl et al.⁸ sectioned the posterior pole horizontally, while Ogden⁵ sectioned it vertically. Varma et al.⁷ divided the optic disc and peripapillary retina into four quadrants, centered on the optic disc, and subsequently shaved sections off the edges of these quadrants, while Quigley and Addicks⁴ trephined (punched out) small circular retinal patches at predetermined locations around the disc, in a hexagonal configuration. In these studies, relatively few eyes were processed, and a limited number of RNFL thickness values were obtained for each of these eyes. The lack of a standardized sectioning and processing approach, and the different species studied (human and primate eyes) all limit comparisons across studies and highlight the technical difficulties involved.

In an attempt to standardize the way in which the RNFL might be analyzed histologically, let us consider the following problem: Assuming that a comprehensive, high resolution, RNFL thickness map is obtainable histologically for a particular eye, which portions of this map would deserve the most attention in relevance to glaucoma diagnosis and staging?

Knowledge gained from RNFL imaging (including confocal scanning laser ophthalmoscopy, scanning laser polarimetry, and optical coherence tomography [OCT]^{1,9}) suggests that much can be learned by analyzing the RNFL thickness values along a predetermined ring set at a specific distance from the optic disc margin. Figure 1A shows a scanning laser polarimetry (GDx-VCC; Laser Diagnostics Technologies, San Diego, CA) RNFL thickness map of a 20° × 20° (roughly 7 × 7 mm) area centered on the optic disc. Although this map contains a large amount of relevant RNFL thickness data, the analysis and parameters routinely calculated stem primarily from RNFL thicknesses located along a peripapillary measurement ring with an outer edge diameter of 3.2 mm (Fig. 1B). Likewise, the OCT (Carl Zeiss Meditec, Dublin, CA) measurement ring is set at 3.4 mm.

Similarly, the technique presented in this report generates high-resolution RNFL thickness data collected along a peripapillary ring placed at a set distance from the optic disc margin. The approach presented is designed to provide comprehensive data on the overall status of the RNFL in the processed eye, on a single histologic section.

METHODS

Globes

Two eyes from two healthy adult pigs were harvested within 30 minutes of death, at a meat farm, and were immediately fixed with 4% buffered formaldehyde. Institutional approval from the Research on Animals committee was obtained.

Two human eyes—a right eye from a 60-year-old white male and a left eye from a 58-year-old white male—were obtained for research purposes from an eye bank. These eyes were fixed in 4% buffered formaldehyde 10.5 and 4.5 hours after death, respectively. Consent for the use of tissues in medical research was obtained

From the Department of Ophthalmology, Hebrew University, Hadassah Medical Center, Jerusalem, Israel.

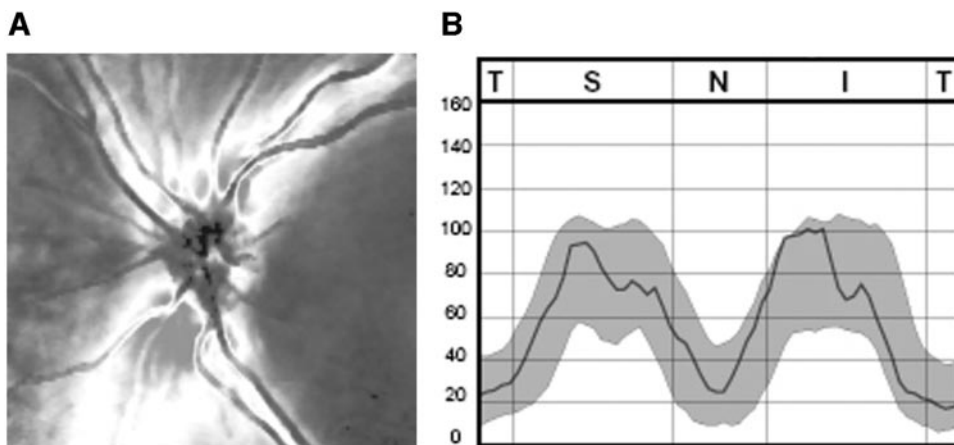
Submitted for publication July 16, 2003; revised August 31, 2003; accepted September 10, 2003.

Disclosure: **E.Z. Blumenthal**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Eytan Z. Blumenthal, Department of Ophthalmology, Hadassah University Hospital, PO Box 12000, Jerusalem 91120, Israel; eblumenthal@md.huji.ac.il.

FIGURE 1. (A) A scanning laser polarimetry map of a normal eye, providing color-coded RNFL thicknesses in a $20^\circ \times 20^\circ$ area centered on the optic disc. The statistical algorithm chosen in scanning laser polarimetry, as well as with most other RNFL imaging devices, is to analyze a subset of thickness values along a ring of fixed diameter, centered on the optic disc. Most diagnostic parameters derived from this scan ignore all available data points except those RNFL thickness points located along the measurement ring. (B) A typical double-hump pattern observed in normal eyes when the RNFL thickness is plotted 360° along a 3.2-mm diameter measurement ring.



from the immediate family. Tissue harvesting was performed according to the regulations for obtaining and handling human tissue for research purposes and in accordance with the tenets of the Declaration of Helsinki for research involving human subjects. Approval for this study was obtained from the institutional Human Subject Committee. A detailed postmortem history was obtained, in which known eye diseases, previous eye surgery, chronic eye medications, diabetes or any other systemic diseases that may affect retinal morphology were all denied. Macroscopic examination of the eyes excluded gross disease.

Sectioning the Globes

The globes were initially opened anterior to the equator, but posterior to the ora serrata, such that the optic disc was approximately at the center of the posterior cup. Portions of the vitreous were mechanically removed when necessary, and the neurosensory retina was then gently separated from the underlying retinal pigment epithelium with a blunt spatula, from the periphery toward the disc. Occasional chorioretinal adhesions were gently dehisced, avoiding tears in the neurosensory retina, rarely with portions of the RPE remaining adherent to the neurosensory retina. Figure 2 illustrates the “umbrella” technique developed for this study, transforming a relatively flat (spherical) configuration of the peripapillary retina into a closed-funnel orientation. This three-dimensional transformation is the core maneuver underlying the new technique. Optic disc orientation was maintained by leaving a rim of sclera around it.

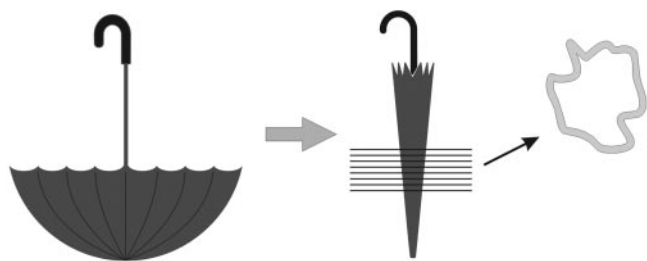


FIGURE 2. The umbrella technique: an illustration demonstrating the transformation of the in vivo two-dimensional (actually concave) structure of the peripapillary retina into a three-dimensional closed-umbrella configuration. Sectioning the retina in such a configuration results in concentric ring sections of gradually increasing diameter. In each of these ring sections the axons composing the RNFL are sectioned perpendicularly to each axon’s long-axis orientation, at an identical distance from the disc margin. Each concentric ring section contains all axons of that eye.

Processing the Tissue

Once maneuvered and stabilized in the desired orientation, the formalin-fixed tissue was processed and embedded in paraffin. The scleral rim surrounding the disc served to orient the tissue perpendicularly within the paraffin block, assuming a closed funnellike configuration. Serial $3\text{-}\mu\text{m}$ sections perpendicular to the long axis of the umbrella were made. The resultant sections, demonstrating circumpapillary rings, were stained with hematoxylin-eosin.

Image Analysis

The ring sections used for RNFL thickness quantification were digitally photographed through a standard light microscope. Multiple high-magnification photographs were taken successively along each ring section, later manually aligned to form a composite image of each histologic slide with higher resolution than would have been possible had the entire slide been photographed at once. A 1-mm bar accompanied each composite photograph, providing a true measurement scale at the histologic section plane. Each digital composite image was later processed with an analysis software program developed in-house for digitally extracting RNFL thickness data at numerous equidistant locations around the inner circumference of each ring section. Measurements were taken on screen using digital calipers (CorelDraw, ver.11; Corel Corp., Ottawa, Ontario, Canada) after caliper calibration against the 1-mm bar. A second operator rechecked all measurements.

Retinal orientation for the human sections was extrapolated from the combined knowledge of the eye (RE versus LE), the orientation in which the block was sectioned (from the disc outwards), identification of the two temporal vascular arcades, and identification of the macula, showing a multilayered ganglion-cell layer region. For the human sections, the precise circumference along the inner retinal border was digitally measured, and then, at equidistant locations along this circumference line, RNFL thickness measurements were obtained with a digital on-screen caliper. Measurements were taken perpendicular to the retinal surface. The RNFL edges were defined such that the innermost edge of the RNFL was determined as the inner limiting membrane, whereas the outer edge was localized as the line joining the innermost extent of the nuclei of the innermost layer of ganglion cells. In the event that the RNFL layer was split, a correction for the empty space was made, such that the final measurement corresponded to actual RNFL tissue, digitally subtracting any empty spaces resulting from cleavage within the RNFL layer. However, most often these occasional splits (cleavage planes) occurred between the RNFL layer and the ganglion cell layer, so that the RNFL layer remained intact. In areas

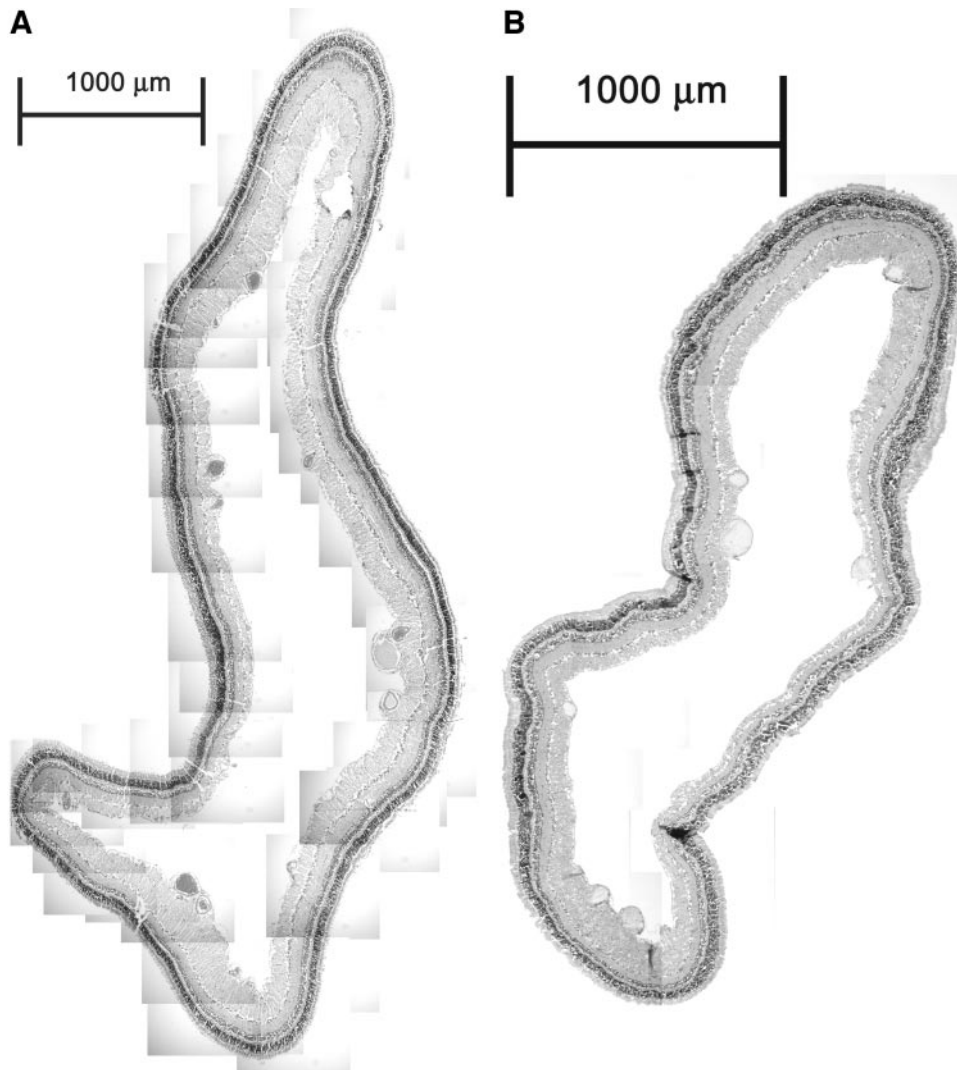


FIGURE 3. Ring sections from two porcine eyes, demonstrating the RNFL distribution in this species. These ring sections, centered on the optic disc, measure 3.79 and 2.29 mm in diameter in the (A) and (B) sections, respectively.

in which a major blood vessel interrupted the RNFL tissue, a RNFL measurement was not taken, but was replaced instead with the numerical average of the RNFL thickness measurements from the two adjacent data points.

RESULTS

Figure 3 shows ring sections from two pig eyes. Figure 4 shows ring sections from two normal human eyes. These ring sections include the entire 360° circumference along the peripapillary retina, centered on the optic disc, at a set diameter around the disc. Each ring section contains all axons of that eye, sectioned perpendicular to each axon's long axis, at a set distance from the optic disc margin. Analyzing a single ring section can provide data quantifying the status of the RNFL in that particular eye, as well as providing information on other neurosensory retina layers. Figure 5 contains data measured from the human ring-sections in Figure 4, presented as a 360° peripapillary RNFL thickness graph, in a format analogous to the scanning laser polarimetry graph presented in Figure 1B.

During the maturation of this histologic technique, several artifacts and issues were encountered. It took several dozen porcine eyes to overcome obstacles related

to dissecting the peripapillary retina; determining the orientation; ascertaining perpendicularity, tissue collapse, tears, and disruption during dissection and processing; stabilizing the tissue orientation; and sectioning. Figure 6 demonstrates some of these artifacts, including nonperpendicular oblique sectioning (Fig. 6A), detachment of the inner limiting membrane (Fig. 6B), and "splitting" of the retina layers parallel to the surface (Fig. 6C), mainly encountered at the RNFL-ganglion cell layer interface.

DISCUSSION

Histologic validation of RNFL thickness may be considered as the gold standard against which any imaging technology should be compared.⁶ More so, a histologic end point that can identify focal glaucomatous RNFL dropout may have advantages over total optic nerve axonal counts^{10,11} as a gold standard for evaluating potential glaucoma therapies in animal models, regardless of whether this treatment is directed at lowering intraocular pressure, improving vascular perfusion, or neuroprotection. As focal changes are a hallmark of glaucoma, both in optic disc assessment

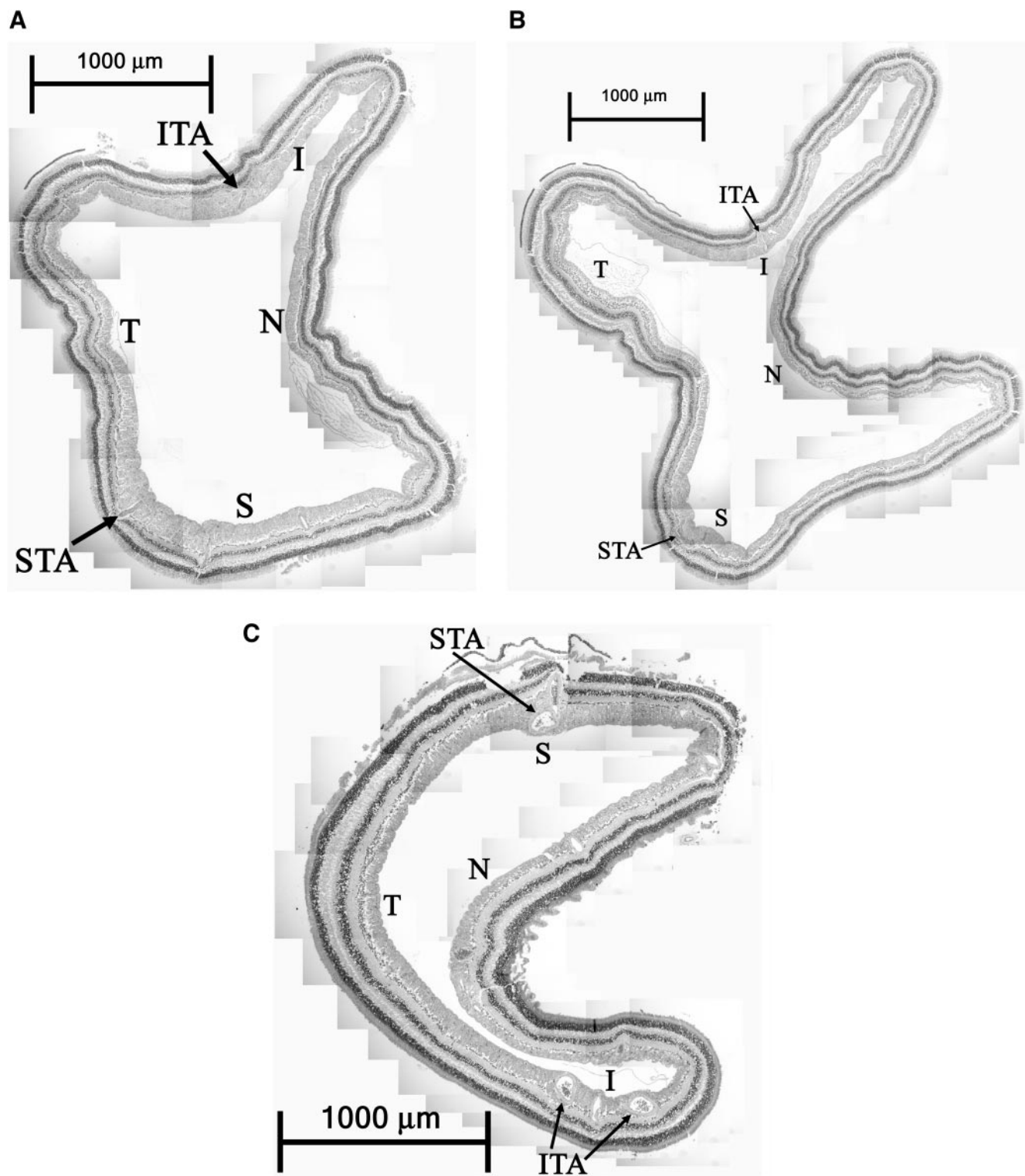


FIGURE 4. Ring sections from two normal human eyes. Concentric sections demonstrate the typical double-hump pattern distribution of the RNFL thickness along a peripapillary ring. *Arrows:* superotemporal arcade (STA) and inferotemporal arcade (ITA) vessels. **(A)** Subject 1: a 2.67-mm diameter ring section centered on the optic disc. **(B)** Subject 1: a 4.29-mm diameter ring section centered on the optic disc. Note that the temporal aspect of the section has entered the macular area, as evident by a multilayered ganglion cell layer. **(C)** Subject 2: a 2.24-mm diameter ring section centered on the optic disc. T, temporal; S, superior; N, nasal; I, inferior.

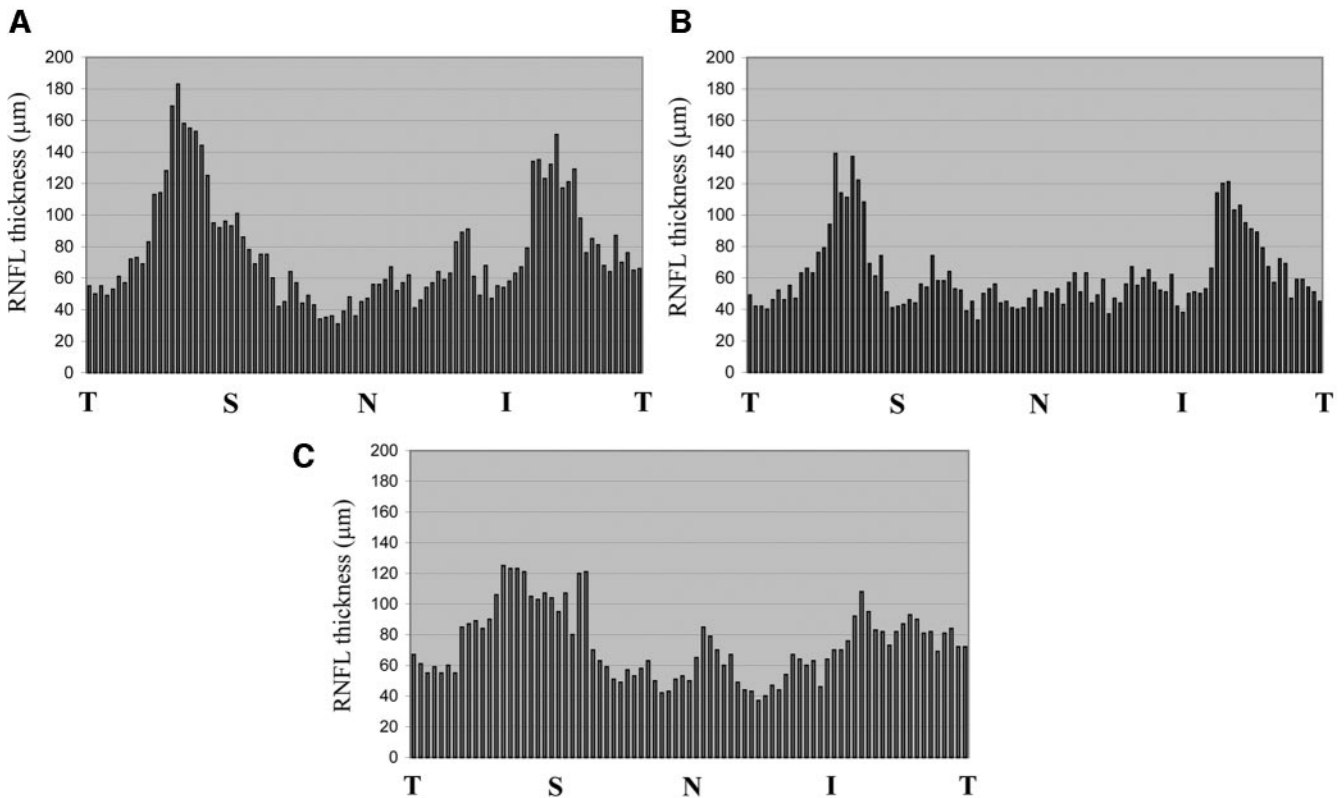


FIGURE 5. RNFL thicknesses mapping RNFL thickness in the three ring sections shown in Figure 4. (A) A thickness graph extracted from the section in 4A, including 94 measured data points. (B) A thickness map extracted from the section in 4B, including 101 measured data points. (C) A thickness map extracted from the section in 4C, including 81 measured data points.

and in visual field interpretation, identifying focal changes histologically may be superior to a global assessment approach.

The umbrella technique described in this report provides ring sections of the peripapillary retina, each providing all the necessary information for quantifying the RNFL layer of that eye, on a single histologic slide. Stated otherwise, this single peripapillary ring section contains all axons of that eye, each sectioned perpendicular to its long axis, at a specified set distance from the optic disc margin, providing the means to quantify focal RNFL dropout in a standardized and familiar layout. To be precise, all axons of the eye are included in the ring section, except the axons of those few ganglion cells with cell bodies that lie within the boundaries of the ring itself.

The umbrella technique presented in this report is not limited in the choice of section thickness, staining chosen, or type of microscopy used. Also, in addition to RNFL analysis, it can serve to determine and quantify various characteristics of other layers of the neurosensory retina.

Several limitations should be mentioned regarding the ability of this novel tissue processing technique to quantify RNFL thickness, limitations that are mostly common to all histologic analyses of the RNFL. These include postmortem and tissue-processing artifacts, such as swelling, shrinkage, expansion, distortion, and autolysis. In addition, several limitations are specific to the umbrella technique—among them, the need to ascertain that the

funnel-like retina configuration is indeed tight and that perpendicularity is maintained during processing. The importance of verifying perpendicularity of the sectioning plane, as well as potential tissue distortion, collapse, and breakage from mobilizing fixated tissue, are all crucial. Ring-sections at the disc margin and very adjacent to it, are difficult to obtain using this technique. Retinal folds during processing that are oriented parallel to the disc margin may result in inaccurate topographic representation. Although the tissue-processing technique minimizes the occurrence of such folds, this can be further studied by comparing the distance of blood vessel bifurcations from the disc margin in the fundus photograph, to their location in successive histologic sections. Last, and perhaps most important, the unique approach presented herein necessitates that the entire eye be dedicated to this sectioning approach, making it difficult to combine this method with other more conventional posterior segment histologic sectioning approaches or to use tissue sectioned previously.⁸

Future goals related to this newly introduced technique include standardizing the processing, sectioning, and analysis stages; validating the reproducibility of this new technique; determining normative values for RNFL thickness in normal human eyes; using it as a histologic endpoint for glaucoma research in animal models; and exploring ways of counting RNFL axons and measuring axonal diameter distribution at the retinal level.

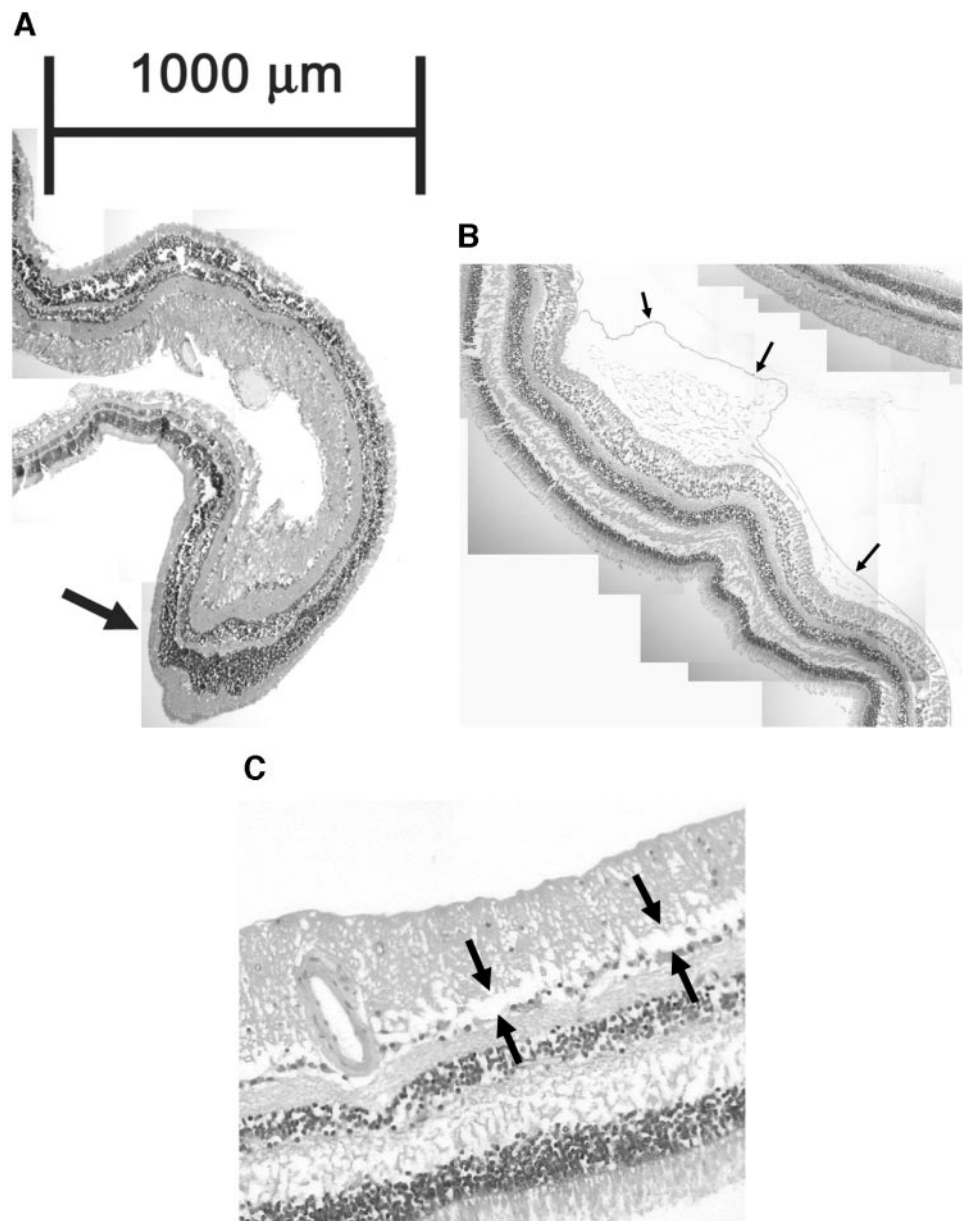


FIGURE 6. Several artifacts are encountered with this technique. (A) nonperpendicular (oblique) section demonstrating thickening of all retinal layers (arrow). (B) Detachment of the inner limiting membrane (arrows), which does not seem to affect the integrity of the RNFL. (C) Splitting between retinal layers (arrows), parallel to the surface, mainly occurring at the RNFL-ganglion cell layer interface. This postmortem artifact may stem from weakening of the tissue by postmortem autolysis, from mechanical tension and shearing forces exerted during processing, or from a combination of the two.

References

- Blumenthal EZ, Weinreb RN. Assessment of the retinal nerve fiber layer in clinical trials of glaucoma neuroprotection. *Surv Ophthalmol.* 2001;45(suppl 3):S305-S312.
- Zangwill LM, Williams JM, Weinreb RN. Quantitative methods for evaluating the retinal nerve fiber layer in glaucoma: lasers in ophthalmology. *Ophthalmol Clin North Am.* 1998;11:233-241.
- Radius RL. Thickness of the retinal nerve fiber layer in primate eyes. *Arch Ophthalmol.* 1980;98:1625-1629.
- Quigley HA, Addicks EM. Quantitative studies of retinal nerve fiber layer defects. *Arch Ophthalmol.* 1982;100:807-814.
- Ogden TE. Nerve fiber layer of the primate retina: thickness and glial content. *Vision Res.* 1983;23:581-587.
- Morgan JE, Waldock A, Jeffery G, Cowey A. Retinal nerve fibre layer polarimetry: histological and clinical comparison. *Br J Ophthalmol.* 1998;82:684-690.
- Varma R, Skaf M, Barron E. Retinal nerve fiber layer thickness in normal human eyes. *Ophthalmology.* 1996;103:2114-2119.
- Dichtl A, Jonas JB, Naumann GO. Retinal nerve fiber layer thickness in human eyes. *Graefes Arch Clin Exp Ophthalmol.* 1999; 237:474-479.
- Zangwill LM, Bowd C, Berry CC, et al. Discriminating between normal and glaucomatous eyes using the Heidelberg Retina Tomograph, GDx Nerve Fiber Analyzer, and Optical Coherence Tomograph. *Arch Ophthalmol.* 2001;119:985-993.
- Yucel YH, Gupta N, Kalichman MW, et al. Relationship of optic disc topography to optic nerve fiber number in glaucoma. *Arch Ophthalmol.* 1998;116:493-497.
- Mikelberg FS, Drance SM, Schulzer M, Yidegiline HM, Weis MM. The normal human optic nerve: axon count and axon diameter distribution. *Ophthalmology.* 1989;96:1325-1328.