

High-Resolution Imaging of Retinal Nerve Fiber Bundles in Glaucoma Using Adaptive Optics Scanning Laser Ophthalmoscopy

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- **PURPOSE:** To detect pathologic changes in retinal nerve fiber bundles in glaucomatous eyes seen on images obtained by adaptive optics (AO) scanning laser ophthalmoscopy (AO SLO).
- **DESIGN:** Prospective cross-sectional study.
- **METHODS:** Twenty-eight eyes of 28 patients with open-angle glaucoma and 21 normal eyes of 21 volunteer subjects underwent a full ophthalmologic examination, visual field testing using a Humphrey Field Analyzer, fundus photography, red-free SLO imaging, spectral-domain optical coherence tomography, and imaging with an original prototype AO SLO system.
- **RESULTS:** The AO SLO images showed many hyperreflective bundles suggesting nerve fiber bundles. In glaucomatous eyes, the nerve fiber bundles were narrower than in normal eyes, and the nerve fiber layer thickness was correlated with the nerve fiber bundle widths on AO SLO ($P < .001$). In the nerve fiber layer defect area on fundus photography, the nerve fiber bundles on AO SLO were narrower compared with those in normal eyes ($P < .001$). At 60 degrees on the inferior temporal side of the optic disc, the nerve fiber bundle width was significantly lower, even in areas without nerve fiber layer defect, in eyes with glaucomatous eyes compared with normal eyes ($P = .026$). The mean deviations of each cluster in visual field testing were correlated with the corresponding nerve fiber bundle widths ($P = .017$).
- **CONCLUSIONS:** AO SLO images showed reduced nerve fiber bundle widths both in clinically normal and abnormal areas of glaucomatous eyes, and these abnormalities were associated with visual field defects, suggesting that AO SLO may be useful for detecting early nerve fiber bundle abnormalities associated with loss of

visual function. (Am J Ophthalmol 2013;155:870–881. © 2013 by Elsevier Inc. All rights reserved.)

EVALUATION OF THE NERVE FIBER LAYER (NFL) is important for detecting and managing glaucoma. However, it has been reported that NFL defects could not be visualized until NFL thickness at the center of the NFL defect decreased to less than 50% of the normal value in experimental primates.¹ It also is difficult to obtain fundus photographs with sufficient quality for interpretation, especially for eyes with a hypopigmented fundus or myopia, when background reflection is high and contrast is low. The advent of optical coherence tomography (OCT) enabled cross-sectional imaging of the NFL, improving detection of damage to the NFL and allowing measurement of the NFL thickness. Relatively high diagnostic sensitivity and specificity for glaucoma detection has been demonstrated for circumpapillary NFL thickness using time-domain OCT and spectral-domain (SD) OCT.^{2–6}

The NFL comprises mainly nerve fiber bundles and Müller cell septa.^{7–10} A nerve fiber bundle has the form of a square rod with 3 dimensions: width, height, and length. The NFL thickness measured with OCT represents the height of the nerve fiber bundles, but not their width. Neither red-free fundus photography nor OCT can provide sufficiently clear images of individual nerve fiber bundles. Thus, the width of nerve fiber bundles, particularly the involvement of structural abnormalities in glaucoma, has not been assessed.

The OCT and other imaging methods such as scanning laser ophthalmoscopy (SLO) fail to provide sufficiently detailed images of NFL microstructure, primarily because of aberrations in ocular optics. These aberrations can be compensated for by using imaging systems that incorporate adaptive optics (AO), consisting of a wavefront sensor that measures aberrations in ocular optics and a deformable mirror or a spatial light modulator to compensate for these aberrations in living eyes.¹¹ Adding AO to imaging systems such as flood-illuminated ophthalmoscopes, SLO equipment, or OCT has allowed researchers to identify individual cone photoreceptors,^{11–25} nerve fiber bundles,²⁶ and blood flow.²²

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Recently, we demonstrated that AO SLO can depict individual retinal nerve fiber bundles in the macula and around the optic disc in normal eyes; the hyperreflective bundles on AO SLO represent retinal nerve fiber bundles, and the dark lines among the hyperreflective bundles on AO SLO represent Müller cell septa.²⁶ In the present study, we used an AO SLO system developed by the authors to conduct high-resolution imaging of the NFL around the optic disc in eyes with open-angle glaucoma and healthy controls to identify structural abnormalities in individual retinal nerve fiber bundles and compared the pathologic changes we saw with abnormalities on images obtained by other methods and with abnormalities in these patients' visual function.

METHODS

• **PARTICIPANTS:** Candidates in this prospective, cross-sectional study were patients with open-angle glaucoma who visited the Kyoto University Hospital, Kyoto, Japan, between April 2010 and August 2011 and agreed to participate in the study, as well as healthy volunteers. All the investigations in this study adhered to the tenets of the Declaration of Helsinki, and this prospective study was approved by the Institutional Review Board and the Ethics Committee of Kyoto University Graduate School of Medicine. The nature of the study, participation in its research, and its possible consequences were explained to the study candidates, after which written informed consent was obtained from all participants.

• **OPHTHALMOLOGIC EXAMINATIONS OF GLAUCOMA PATIENTS AND NORMAL VOLUNTEERS:** All patients and volunteers in this study underwent comprehensive ophthalmologic examinations, including autorefractometry and keratometry, uncorrected and best-corrected visual acuity measurements using a 5-m Landolt chart, intraocular pressure (IOP) using a Goldmann applanation tonometer, axial length assessed using an IOLMaster (Carl Zeiss Meditec, Dublin, California, USA), visual field testing using the Humphrey Field Analyzer (Carl Zeiss Meditec), gonioscopy, dilated funduscopy, stereo fundus photography, red-free SLO fundus imaging, circumpapillary NFL thickness measurement using SD OCT, and AO SLO.

Glaucomatous eyes were defined by the presence of evident diffuse or localized rim thinning on stereo disc photography, regardless of the presence or absence of glaucomatous visual field defects. All of the study eyes already had been classified as glaucomatous during our glaucoma service meeting on the basis of the appearance of the optic discs of both eyes in each patient based on fundus photography, including stereoscopic photography. The optic disc appearance was evaluated independently by 3 glaucoma specialists (M.H., T.A., A.N.) who were masked to all

other information about the eyes. Eyes were classified as having glaucoma if the examiner identified either diffuse or localized rim thinning. If all 3 examiners did not agree with the classification of an eye, the group reviewed and discussed the fundus color photographs and stereo photographs until a consensus was reached.

Visual field defects resulting from glaucoma were defined according to the Anderson and Patella criteria²⁷ using standard automated perimetry and the 24-2 Swedish interactive threshold algorithm standard as follows: (1) abnormal range on the glaucoma hemifield test or (2) pattern standard deviation of less than 5% of the normal reference value confirmed on 2 consecutive tests considered reliable based on fixation losses of less than 20%, false-positive results of less than 20%, and false-negative results of less than 20%. The 2 consecutive visual field tests were performed within 1 month of each other, and when the results of these did not agree, a third test was performed in another month. A visual field focal defect was defined as the depression of 3 points to an extent present in less than 5% of the normal population. At least 1 point of these 3 should be depressed to an extent found in less than 1% of the normal population. The sectoring method of Garway-Heath and associates was used for analysis of correlations between visual field indices and nerve fiber bundle widths (Figure 1).²⁸ Area 2 and area 5, corresponding to the nerve fiber bundles running through the areas extending from 271 to 310 degrees (inferior temporal) and 41 to 80 degrees (superior temporal), respectively, were used for the analysis. The mean deviation (MD) for each area was calculated by averaging anti-log values of total deviation values of each point.²⁹

Eyes with a normal open angle but with glaucomatous optic disc appearance were included in this study. Exclusion criteria were as follows: (1) contraindication to dilation; (2) Snellen equivalent best-corrected visual acuity worse than 20/40; (3) spherical equivalent refractive error of more than 5.0 or less than -6.0 diopters or cylindrical refractive error of less than -3.0; (4) unreliable Humphrey Field Analyzer results (fixation loss of $\geq 20\%$, false-positive or false-negative results of $\geq 20\%$); (5) nonglaucomatous visual field defects suggesting brain diseases; (6) history of intraocular surgery; (7) evidence of vitreoretinal diseases; or (8) evidence of brain diseases, diabetes mellitus, or other systemic diseases that may affect the eye.

• **ADAPTIVE OPTICS SCANNING LASER OPHTHALMOSCOPY SYSTEM:** The usefulness of incorporating a wide-field SLO with an AO SLO was reported by Burns and associates and Ferguson and associates.^{30,31} We designed and constructed our AO SLO system based on the same scheme with certain simplifications.^{17-19,26} The AO SLO system comprises 4 primary optical subsystems, the AO subsystem including the wavefront sensor, the high-resolution confocal SLO imaging subsystem, the wide-field imaging subsystem, and the pupil observation subsystem for initial alignment of the subject's pupil with

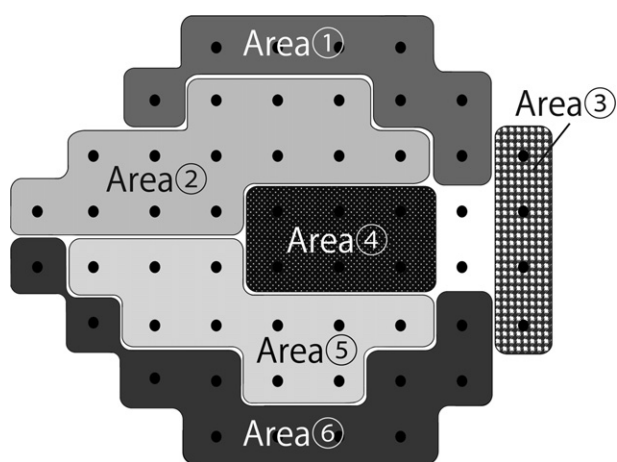


FIGURE 1. Visual field clusters from the Humphrey Field Analyzer 24-2 Swedish interactive threshold algorithm standard program (Carl Zeiss Meditec, Dublin, California, USA). Area 2 corresponds to the nerve fiber bundles that pass through the areas extending from 271 to 310 degrees (inferior temporal), and area 5 corresponds to the areas extending from 41 to 80 degrees (superior temporal).

the optical axis of the AO SLO system by adjusting the chin rest. The details of the current AO SLO system are described in the [Supplemental Material](#) (available at [AJO.com](#)).

• **MEASUREMENT OF WIDTHS OF THE HYPERREFLECTIVE BUNDLES:** Methods for measuring hyperreflective bundle widths using AO SLO imaging have been described elsewhere.²⁶ For each eye, AO SLO images (3.0×1.9 degrees) were obtained at multiple locations around the optic disc (4.5×4.5 mm). All eyes were dilated for examination, and AO SLO imaging was performed by focusing on the surface of the NFL. A montage of AO SLO images then was created offline by selecting the area of interest and generating each image to be included in the montage from a single frame, without averaging. The degree of correspondence of each montage to the area of interest was verified by comparing the AO SLO image with the wide-field images for that eye. To create a large-scale montage of AO SLO images (Figure 2 and Supplemental Figure), an automated image-stitching algorithm was applied.

To measure the width of individual hyperreflective bundles, several bundles were chosen from an AO SLO image. We analyzed avascular areas, because vessels can obscure underlying nerve fiber bundles in AO SLO images. The digital caliper tool built into ImageJ (National Institutes of Health, Bethesda, Maryland, USA) was used to measure the width at 3 points in each bundle by 2 independent experienced graders (S.O. and N.U.-A.) who were masked to the bundle location and other clinical information regarding the eyes. For each area of each eye,

13.2 ± 4.0 points were measured. To obtain accurate scan lengths, we corrected for the magnification effect in each eye using the adjusted axial length method devised by Bennett and associates.³² The width of each hyperreflective bundle was determined as the mean width acquired from these images. If the values were different significantly between the graders, a third grader (K.T.) was invited, and the value closest to that determined by the third grader was selected. The mean value of the 2 independent graders was used as each bundle width.

Measurements of hyperreflective bundle width were performed in 12 AO SLO images obtained at 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, and 330 degrees from the temporal horizontal (clockwise in the right eye and counterclockwise in the left eye) along a circle with a diameter of 3.4 mm.

Only eyes for which adequate image quality was obtained were included in this study, and if both eyes were eligible, 1 eye was randomly selected for the analysis.

• **CIRCUMPAPILLARY NERVE FIBER LAYER THICKNESS MEASUREMENT:** The SD OCT examinations were performed on all eyes using the Spectralis HRA+OCT (Heidelberg Engineering, Dossenheim, Germany). We exported the raw data from the Spectralis HRA+OCT and calculated the mean NFL thickness for each of the 12 areas (0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, and 330 degrees from the temporal horizontal midline [clockwise in the right eye and counterclockwise in the left eye]) along a 3.4-mm diameter circle centered on the optic disc.

• **STATISTICAL ANALYSES:** The best-corrected visual acuity measured using the Landolt chart was expressed as the logarithm of the minimum angle of resolution. For comparing bundle width variables among areas, Bonferroni correction was used. Variables were compared between normal eyes and glaucomatous eyes using a *t* test. For interobserver measurements, 2-way mixed, average measure intraclass correlation coefficients (ICC [3, K]) were obtained. For intraobserver measurements, 1-way random, average measure ICCs (ICC [1, K]) were obtained. Relationships between nerve fiber bundle widths and the visual field tests or circumpapillary NFL thickness were assessed using Pearson correlation analysis. Statistical analyses were performed using the SPSS statistics software program version 17 (SPSS Inc, Chicago, Illinois, USA). A *P* value less than .05 was considered statistically significant.

RESULTS

EIGHTY-FOUR EYES FROM 42 PATIENTS WITH OPEN-ANGLE glaucoma were examined. Among them, 28 eyes were

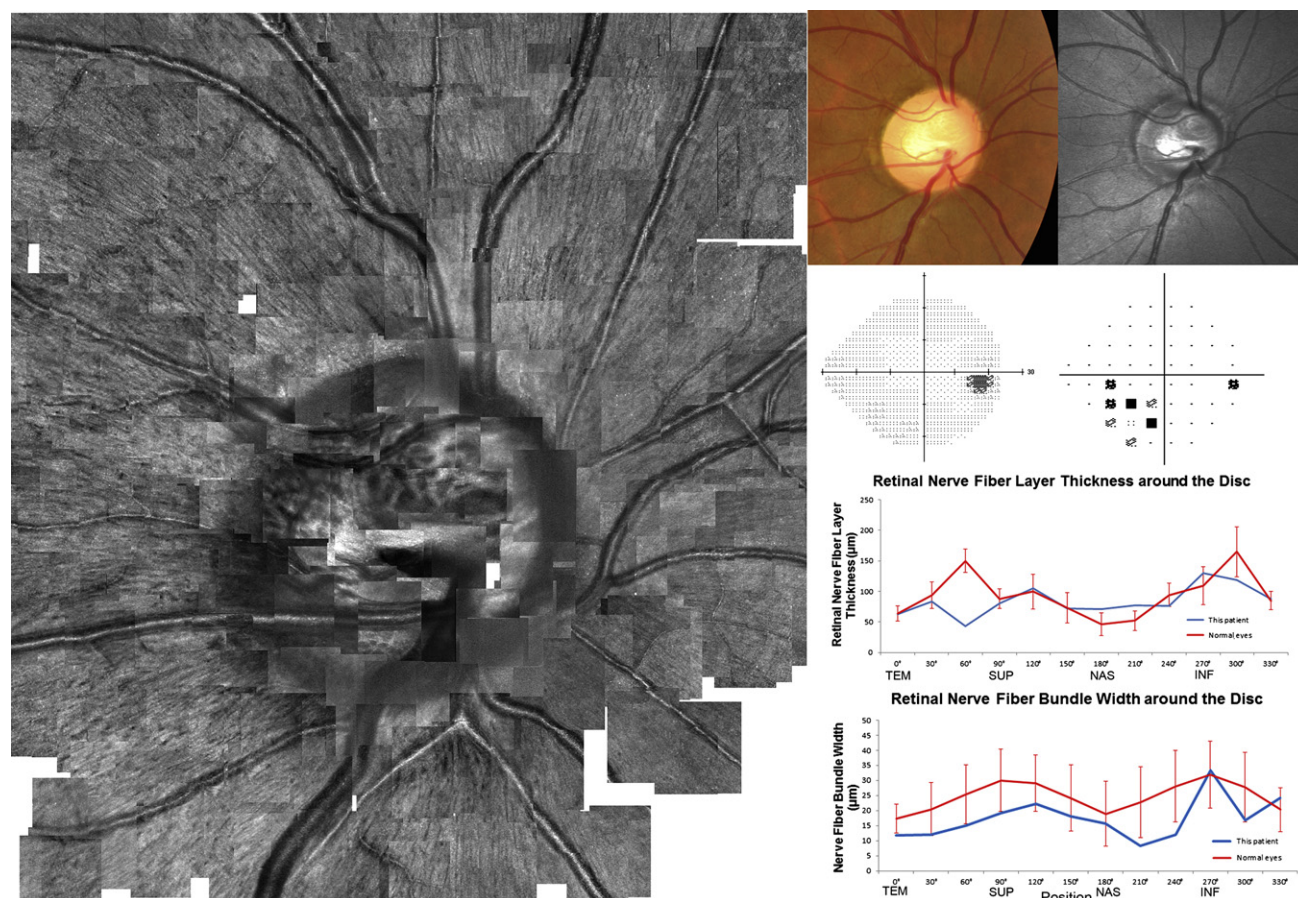


FIGURE 2. High-resolution imaging of retinal nerve fiber bundles in a right eye with glaucoma from a 40-year-old man with normal-tension glaucoma with Snellen equivalent best-corrected visual acuity of 20/12 obtained using adaptive optics scanning laser ophthalmoscopy. (Left) Wide-field montage of adaptive optics (AO) scanning laser ophthalmoscopy (SLO) images. (Top middle) Fundus photograph showing localized neuroretinal rim thinning and nerve fiber layer (NFL) defects in the superior temporal side of the optic disc. (Top right) Red-free SLO image showing the NFL defects more clearly. (Second row) Humphrey Field Analyzer 24-2 Swedish interactive threshold algorithm standard program (Carl Zeiss Meditec, Dublin, California, USA) results. The left image is the gray-scale map and the right image is the total deviation map. The mean deviation was -2.76 dB. (Third row) Circumpapillary NFL thickness measured by spectral-domain optical coherence tomography along a circle with a diameter of 3.4 mm centered on the optic disc. Circumpapillary NFL thickness is decreased at the superior temporal side of the optic disc. (Bottom) Widths of the nerve fiber bundles measured using AO SLO along a circle with a diameter of 3.4 mm centered on the optic disc (blue). Red indicates the mean width of the nerve fiber bundles in normal eyes. Error bars represent 2 standard deviations for 21 normal eyes. INF = inferior; NAS = nasal; SUP = superior; TEM = temporal.

excluded because of the poor image quality (because of media opacity, insufficient dilation, poor fixation, or a combination thereof), and 3 eyes were excluded because of unreliable Humphrey Field Analyzer results. Ultimately, the images obtained for 53 eyes from 28 patients were suitable for analysis. If both eyes were eligible for inclusion, 1 eye was selected randomly. Thus, 28 eyes from 28 patients were included in this study. Twenty-one normal eyes in 21 subjects were included as control. The ages of the subjects ranged from 31 to 73 years (mean $\pm$ standard deviation, 58.9 ± 9.4 years) for patients with glaucoma and from 26 to 83 years (mean $\pm$ standard deviation, 51.4 ± 16.4 years) for normal volunteers ($P = .051$, t test). The axial length

ranged from 22.5 to 27.2 mm (mean $\pm$ standard deviation, 25.1 ± 1.4 mm) in eyes with glaucoma and 22.0 to 27.1 mm (mean $\pm$ standard deviation, 24.5 ± 1.4 mm) in normal eyes ($P = .114$, t test).

Twenty-four (85.7%) eyes had glaucomatous visual field defects corresponding to the evident optic disc rim thinning (perimetric glaucoma), and 4 (14.3%) eyes did not have glaucomatous visual field defects (preperimetric glaucoma). Nineteen (67.9%) eyes had a mean deviation (MD) of -6 dB or more, and 9 (32.1%) eyes had an MD of less than -6 dB. The median MD was -3.77 dB, the first interquartile was -7.79 dB, and the third interquartile was -1.12 dB. The distribution of focal defects in the

visual field was as follows: 14 eyes had visual field defects in area 2, and 16 eyes had visual field defects in area 5. In area 2, the median MD was -3.79 dB, the first interquartile was -5.82 dB, and the third interquartile was -0.875 dB. In area 5, the median of MD was -2.47 dB, the first interquartile was -4.87 dB, and the third interquartile was -1.53 dB.

In all of the eyes, the AO SLO images showed many hyperreflective bundles aligned with the striations on SLO red-free images (Figure 2), suggesting that these structures represent nerve fiber bundles in the NFL. However, the resolution was much higher in the AO SLO images than in fundus photography or red-free SLO images (Figure 2). The visibility of nerve fiber bundles was not associated with disc size.

The reproducibility of the nerve fiber bundle width measurements was evaluated through an interobserver ICC; the ICC was 0.867 for measurement of nerve fiber bundle width in eyes with glaucoma and 0.877 in normal eyes. The 95% confidence interval for ICC values were 0.833 to 0.894 in glaucoma eyes and 0.863 to 0.889 in normal eyes. The ICCs of each quadrant around the disc (temporal, superior, nasal, and inferior) are shown in Table 1.

The mean widths of the nerve fiber bundles along a circle with a diameter of 3.4 mm centered on the optic disc are shown in Figure 3. In normal eyes, the nerve fiber bundles in the temporal and nasal quadrants of the optic disc were narrower than those above and below the optic disc ($P < .001$, Kruskal-Wallis test). Thus, the bundle width around the optic disc had a double-humped shape (Figure 3). Circumpapillary NFL thickness as measured by SD OCT around the optic disc exhibited a similar double-humped shape (Figure 3). The circumpapillary NFL thickness was correlated with corresponding nerve fiber bundle widths on AO SLO images ($P < .001$, $r = 0.374$, Pearson correlation coefficient). In eyes with glaucoma, the nerve fiber bundles were narrower than in normal eyes, especially at 60, 240, and 300 degrees ($P = .014$, $P = .035$, and $P < .001$, respectively; Figure 3 and Table 2). There were significant differences in bundle width at 60, 90, 120, 150, 240, 270, and 300 degrees as compared with the value measured at 0 degrees from the temporal pole of the optic disc in normal eyes ($P < .001$, $P < .001$, $P < .001$, $P = .035$, $P < .001$, $P < .001$, and $P < .001$, respectively). There were significant differences in bundle width at 60, 90, 120, and 270 degrees compared with that measured at 0 degrees from the temporal pole of the optic disc in glaucoma eyes ($P = .040$, $P < .001$, $P = .026$, and $P < .001$, respectively). The circumpapillary NFL thickness was correlated with the corresponding nerve fiber bundle widths on AO SLO ($P < .001$, $r = 0.351$, Pearson correlation coefficient) in eyes with glaucoma.

Changes in the NFL on fundus photography or red-free SLO images (NFL defect) were detectable in 28 areas of

TABLE 1. Intraclass Correlation Coefficients of Measurement of Retinal Nerve Fiber Bundle Width

Area	ICC (95% CI)	
	Interobserver	Intraobserver
Normal eyes		
Total	0.877 (0.863 to 0.889)	0.944 (0.929 to 0.955)
Temporal	0.878 (0.834 to 0.911)	0.835 (0.746 to 0.893)
Superior	0.896 (0.849 to 0.929)	0.915 (0.868 to 0.945)
Nasal	0.880 (0.824 to 0.919)	0.933 (0.893 to 0.959)
Inferior	0.925 (0.892 to 0.948)	0.963 (0.936 to 0.978)
Glaucomatous eyes		
Total	0.867 (0.833 to 0.894)	0.881 (0.857 to 0.902)
Temporal	0.843 (0.779 to 0.889)	0.892 (0.847 to 0.923)
Superior	0.826 (0.735 to 0.886)	0.774 (0.655 to 0.852)
Nasal	0.882 (0.837 to 0.915)	0.907 (0.872 to 0.933)
Inferior	0.943 (0.908 to 0.965)	0.838 (0.738 to 0.900)

CI = confidence interval; ICC = intraclass correlation coefficient.

19 eyes. In 3 of these areas, the nerve fiber bundles were invisible on the AO SLO images (Figure 4). However, AO SLO revealed the nerve fiber bundles remaining in 25 areas (89%) showing NFL defects on fundus photography or red-free SLO imaging (Figures 5 and 6). In the NFL defect area as imaged on fundus photography or red-free SLO imaging, nerve fiber bundle width as measured using the AO SLO ($19.2 \pm 5.1 \mu\text{m}$) was narrower than that observed in normal eyes ($27.4 \pm 5.5 \mu\text{m}$; $P < .001$, t test). There were more hyporeflective areas between the nerve fiber bundles in the NFL defect area in glaucomatous eyes as compared with normal eyes (Table 3). In contrast to glaucoma eyes, there was no focal bundle thinning in any normal eyes.

The nerve fiber bundle width in areas of the retina without NFL defect and visual field defects was narrower in glaucomatous eyes than in normal eyes at 60 degrees on the inferior temporal side of the optic disc ($P = .026$, t test; Table 4).

The sectoring method was used for correlation analysis between the visual field and the bundle widths (Figure 1). Area 2 corresponds to the nerve fiber bundles that run through the areas extending from 271 to 310 degrees (inferior temporal), and area 5 corresponds to the nerve fiber bundles that run through the areas extending 41 to 80 degrees (superior temporal). The MDs for both areas were correlated with the corresponding nerve fiber bundle widths in eyes with glaucoma ($P = .031$, $r = 0.483$, Pearson correlation coefficient).

There were no correlations between mean bundle widths and age, axial length, intraocular pressure, or disc area ($P = .620$, $P = .221$, $P = .101$, and $P = .142$, respectively, and $r = -0.098$, $r = 0.243$, $r = -0.316$, and $r = -0.296$, respectively, Pearson correlation coefficient). There was no

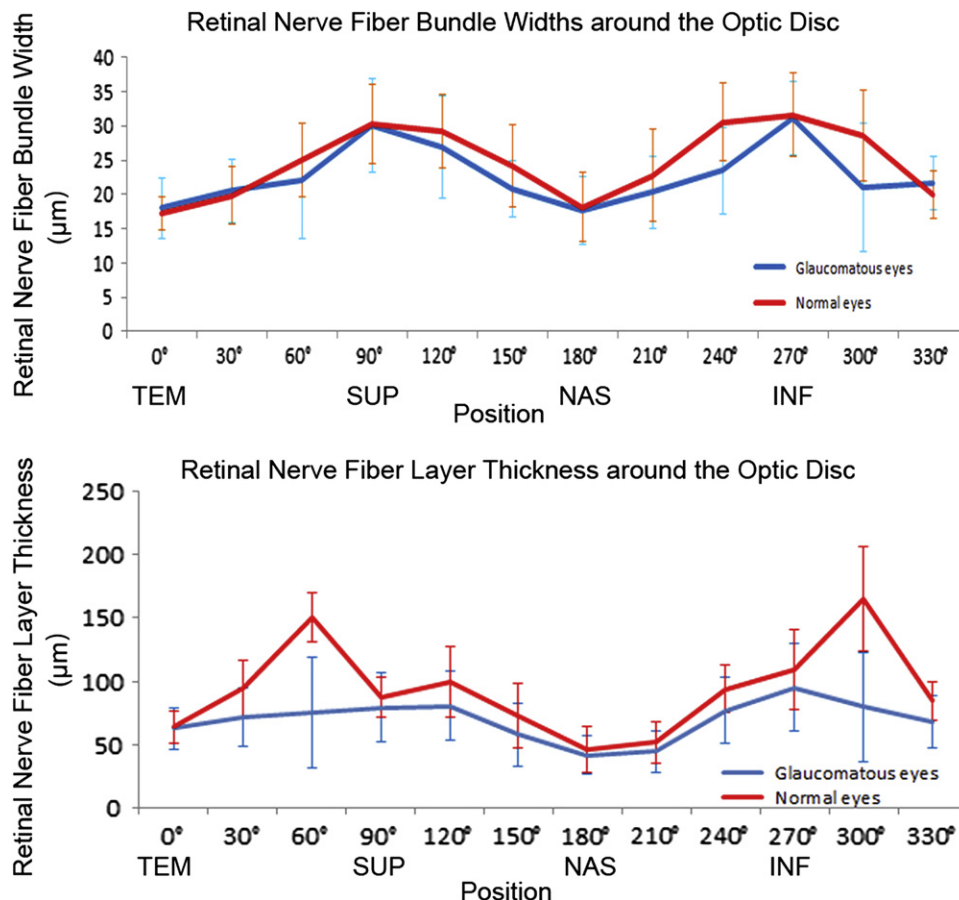


FIGURE 3. Retinal nerve fiber bundle width and retinal nerve fiber layer (NFL) thickness around the optic disc in glaucomatous and normal eyes. (Top) Mean widths of the nerve fiber bundles measured using adaptive optics scanning laser ophthalmoscopy along a circle with a diameter of 3.4 mm centered on the optic disc (blue = glaucoma eyes, red = normal eyes). Error bars represent standard deviations for 21 normal eyes and 28 eyes with glaucoma. (Bottom) circumpapillary NFL thickness measured using spectral-domain optical coherence tomography along a circle with a diameter of 3.4 mm centered on the optic disc. Error bars represent standard deviations for 28 eyes with glaucoma. INF = inferior; NAS = nasal; SUP = superior; TEM = temporal.

difference between men and women in nerve fiber bundle width ($P = .211$, t test).

DISCUSSION

MORPHOLOGIC FEATURES OF NERVE FIBER BUNDLES IN normal eyes have been identified using novel imaging techniques such as AO SLO and AO OCT.^{13,26,33,34} Using AO SLO, we previously reported that hyperreflective bundles at the NFL are retinal nerve fiber bundles.²⁶ Using AO OCT, several researchers have confirmed that the striations seen in C-scan images focused on the NFL are retinal nerve fiber bundles.^{13,33,34} Recently, Kocaoglu and associates performed a pilot study measuring the nerve fiber bundle widths in 4 normal subjects and 1 patient with NFL defect using AO OCT and found that individual nerve fiber bundles were exceedingly thin in the NFL defect,

similar to our results.³⁴ In the current study, we used an AO SLO system to conduct high-resolution imaging of the NFL around the optic disc in eyes with open-angle glaucoma, demonstrating the clinical relevancy of the findings of Kocaoglu and associates.³⁴

In normal eyes in the present study, the bundle width around the optic disc had a double-humped shape similar to the double-humped shape of the circumpapillary NFL thickness, and the circumpapillary NFL thickness on SD OCT was correlated with nerve fiber bundle widths on AO SLO. These findings are consistent with our previous reports.²⁶ This double-hump configuration correlates with the physiologic shape of the neuroretinal rim, which is thickest inferiorly, then superiorly, then nasally, and finally, temporally. In the current study, bundle width exhibited a similar double-humped shape in the area proximal to the optic nerve head. The nerve fiber bundles in the temporal and nasal quadrants of the optic disc were narrower than those above and below the optic disc.

TABLE 2. Retinal Nerve Fiber Bundle Widths around the Optic Disc in Normal versus Glaucoma Eyes

Mean (Standard Deviation) Bundle Width (μm)	Position (Degrees)											
	0	30	60	90	120	150	180	210	240	270	300	330
Normal eyes	17.5 (2.4)	20.5 (4.4)	25.5 (4.9)	30.1 (5.2)	29.2 (4.7)	24.2 (5.5)	19.0 (5.4)	22.8 (5.9)	28.1 (5.9)	31.9 (5.6)	27.9 (5.8)	20.4 (3.6)
Glaucomatous eyes	17.7 (5.1)	19.8 (4.9)	20.3 (6.6)	26.8 (4.5)	26.8 (5.5)	21.5 (4.3)	18.3 (5.2)	18.8 (5.9)	22.3 (6.5)	30.9 (5.7)	19.3 (8.2)	20.8 (2.9)
<i>P</i> value ^a	.878	.658	.014	.166	.349	.397	.784	.198	.035	.695	<.001	.771
^a <i>t</i> test.												

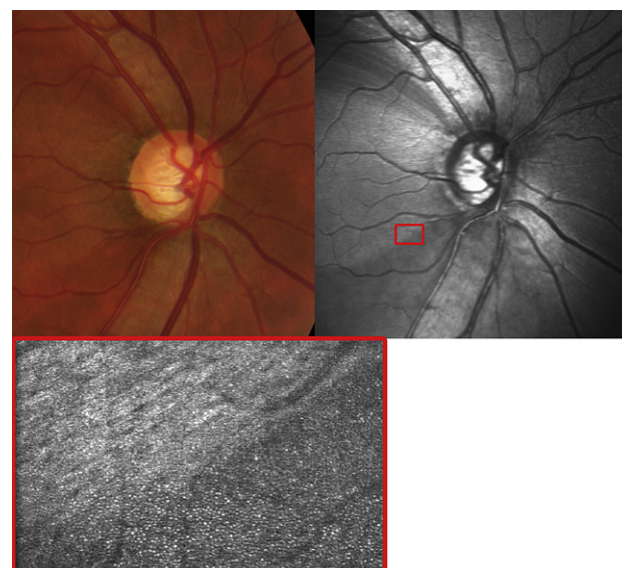
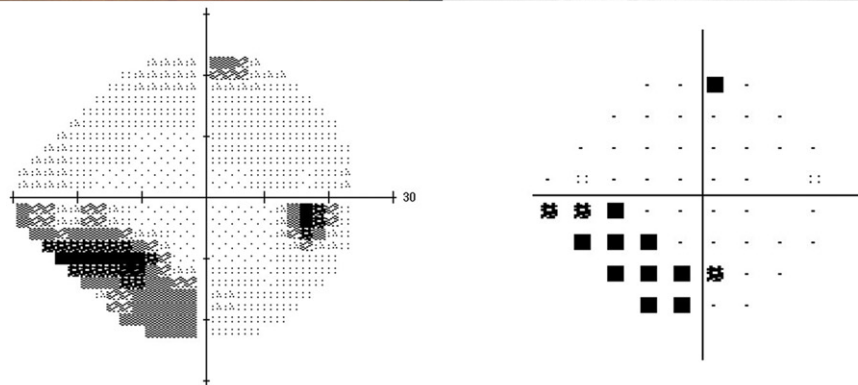
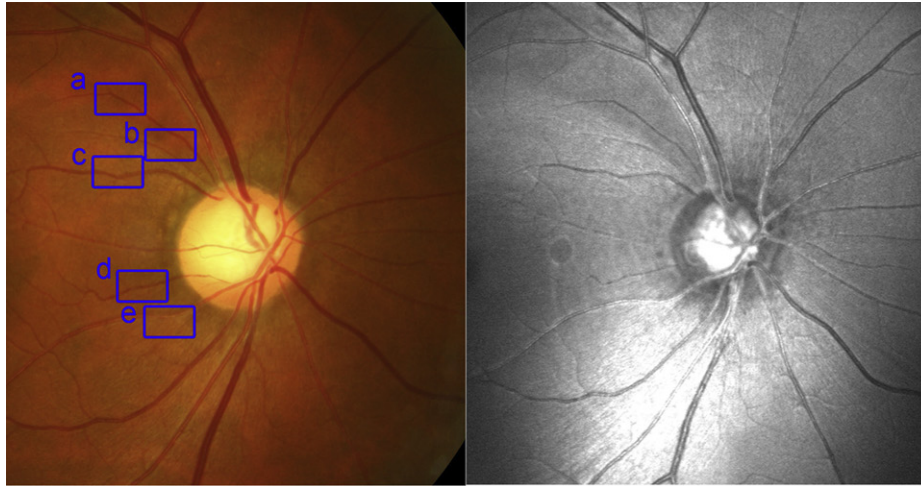


FIGURE 4. Images obtained at the border of the retinal nerve fiber bundle defect from the right eye of a 45-year-old man with primary open-angle glaucoma with Snellen equivalent best-corrected visual acuity of 20/12. (Top left) Fundus photography showing localized neuroretinal rim thinning and nerve fiber layer (NFL) defects in the superior temporal and inferior temporal sides of the optic disc. (Top right) Red-free scanning laser ophthalmoscopy (SLO) image showing the margins of the NFL defects more clearly. (Bottom) High-magnification adaptive optics SLO image focused on the NFL in the area indicated by the red box in the Top right. Nerve fiber bundles are invisible in the area corresponding to NFL defects on fundus photography or the red-free SLO image, and the bare cone mosaic is visible.

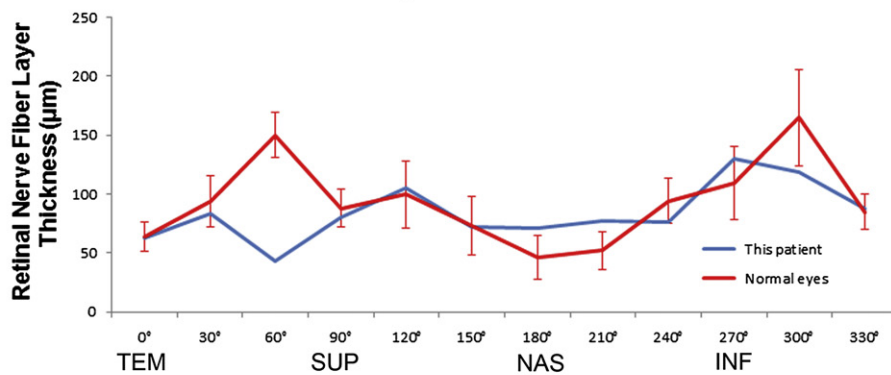
Thus, the double-humped shape and regional differences in neuroretinal rim width may be attributable, at least in part, to the double-hump pattern and regional differences in bundle width, respectively.

In the current study, in eyes with glaucoma, the bundle width around the optic disc also had a double-humped shape, but the nerve fiber bundles were narrower than in normal eyes, especially in the superior temporal (60 degrees), inferior nasal (240 degrees), and inferior temporal (300 degrees) areas. These areas are the same areas in which NFL defects are likely to be observed.³⁵ In addition, the NFL thickness on SD OCT was correlated with nerve fiber bundle widths on AO SLO even in eyes with glaucoma, suggesting that the nerve fiber bundle width may change in proportion to its thickness in eyes with glaucoma.

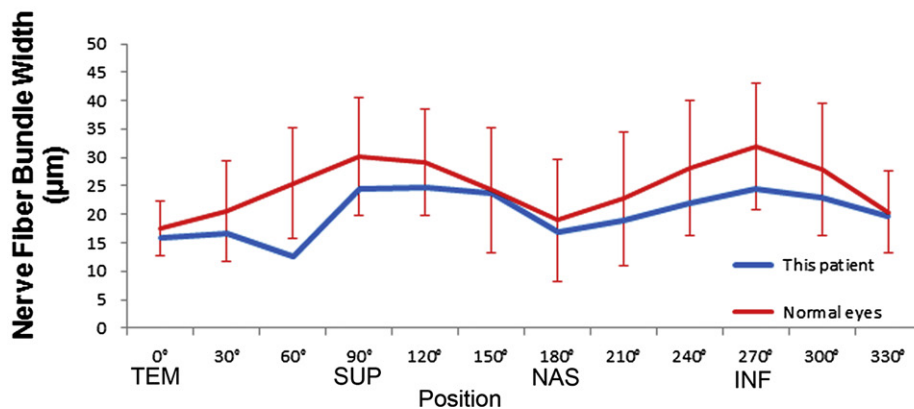
It has been reported that early stage IOP-induced glaucoma damage can involve axon swelling because of impaired axoplasmic flow.^{36,37} However, these axonal changes are acute effects of IOP-induced experimental glaucoma. Measurements of axonal density were reduced at 7 and 14 days. The current study included patients at



Retinal Nerve Fiber Layer Thickness around the Disc



Retinal Nerve Fiber Bundle Width around the Disc



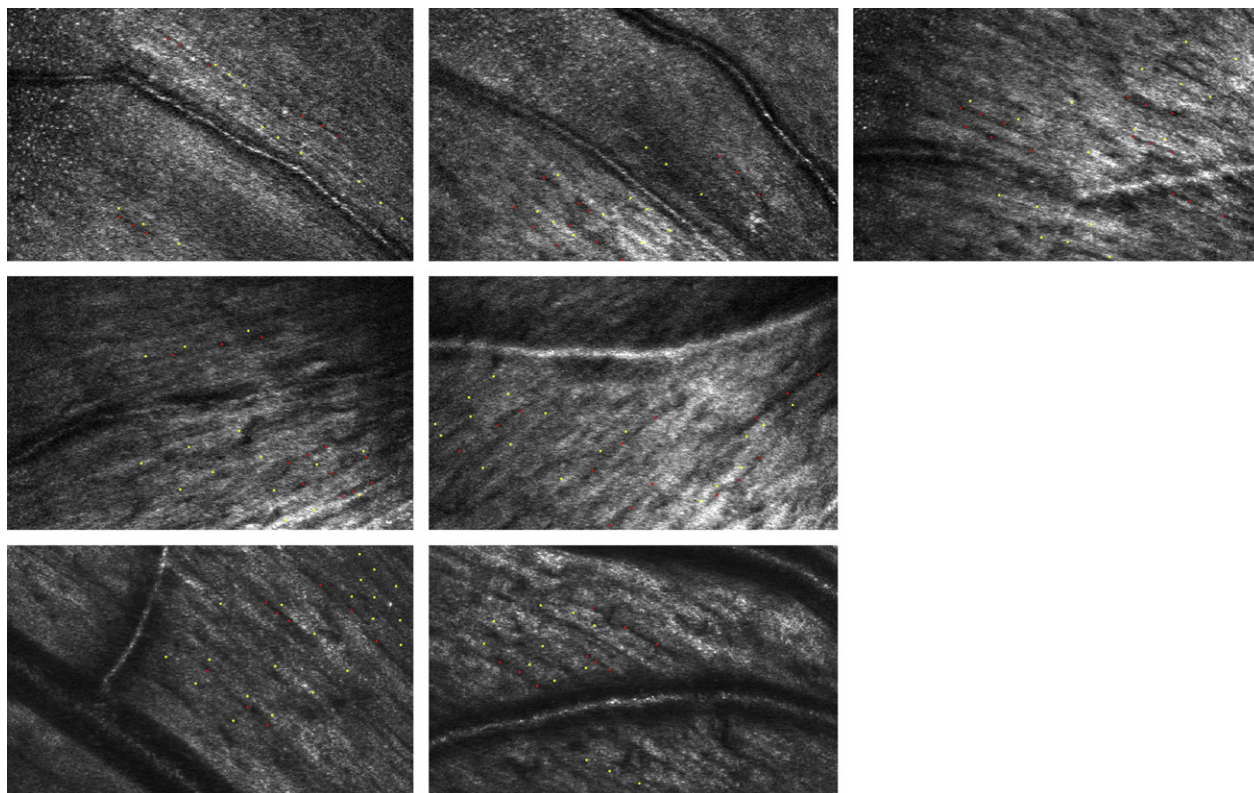


FIGURE 6. High-resolution imaging of retinal nerve fiber bundles in an eye with glaucoma and a normal eye. High-magnification adaptive optics (AO) scanning laser ophthalmoscopy (SLO) images in the area indicated by a, b, c, d, and e in Figure 6 (Top left, a; Top middle, b; Top right, c; Second row left, d; Second row right, e). (Bottom left) Magnified AO SLO image in a normal eye corresponding to the area shown in the Top left. (Bottom right) Magnified AO SLO image in a normal eye corresponding to the area shown in the Top middle. Note that nerve fiber bundles (yellow) are visible even in the nerve fiber layer (NFL) defect area (Top left and Top middle), but narrow in width compared with an area outside the NFL defects in the same hemifield (Top right), an area in the opposite hemifield (Second row right), and in normal eyes (Bottom). There are more hyporeflective areas (red) between the nerve fiber bundles in the NFL defect area in the glaucoma eye compared with the normal eye.

the chronic phase of open-angle glaucoma (mean IOP, 16.4 ± 3.1 mm Hg), so the nerve fiber bundles were considered to comprise fewer axons because of glaucoma-related changes. Thus, the stage of glaucoma insult may play a role in the morphologic appearance of axons, as well as whether an IOP-dependent or IOP-independent component is more relevant in the sample analyzed.

The AO SLO revealed nerve fiber bundles remaining in many areas (89%) in which NFL defects were observed on

fundus photography or red-free SLO imaging. In NFL defect areas on fundus photography or red-free SLO imaging, the nerve fiber bundles on AO SLO were narrower compared with those of normal eyes. These results suggest that NFL defects seen on fundus photography or red-free SLO imaging may not be actual nerve fiber defects, but rather nerve fiber bundle narrowing. Several researchers have reported that in more than 50% of NFL defects detected on red-free fundus images, the NFL on SD OCT

FIGURE 5. Retinal nerve fiber layer (NFL) thickness and retinal nerve fiber bundle width surrounding the optic disc in a right eye with glaucoma from a 63-year-old man with primary open-angle glaucoma with Snellen equivalent best-corrected visual acuity of 20/12. (Top left) Fundus photograph showing localized neuroretinal rim thinning and NFL defects in the superior temporal side of the optic disc. Small blue boxes (a, b, c, d, and e) indicate the area of high-magnification adaptive optics (AO) scanning laser ophthalmoscopy (SLO) images in Figure 6. (Top right) Red-free SLO image showing NFL defects clearly. (Second row) Humphrey Field Analyzer 24-2 Swedish interactive threshold algorithm standard program (Carl Zeiss Meditec, Dublin, California, USA) results. The left image is gray-scale image and the right image is a pattern deviation map. The mean deviation was -5.37 dB. (Third row) NFL thickness measured using spectral-domain optical coherence tomography along a circle with a diameter of 3.4 mm centered on the optic disc. (Bottom) Widths of the nerve fiber bundles measured using AO SLO along a circle with a diameter of 3.4 mm centered on the optic disc (blue). Red indicates the mean width of the nerve fiber bundles in normal eyes. Error bars represent 2 standard deviations of 21 normal eyes. Nerve fiber bundle width is decreased in the superior temporal side, which corresponds to the area with NFL defects on red-free SLO and visual field defects. INF = inferior; NAS = nasal; SUP = superior; TEM = temporal.

TABLE 3. Hyporeflective Area Width in Nerve Fiber Layer Defect Areas and in Normal Eyes

	NFL Defect Area	Normal Eyes	P Value ^a
60 degrees, superior temporal side of the optic disc	14.7 ± 4.2	9.2 ± 2.9	<.001
60 degrees inferior temporal side of the optic disc	13.6 ± 4.8	9.8 ± 3.2	<.001

NFL = nerve fiber layer.
^at test.

TABLE 4. Retinal Nerve Fiber Bundle Width in Areas without Nerve Fiber Layer Defects and Visual Field Defects

	Glaucoma Eyes (μm), (No. of Eyes)	Normal Eyes (μm), (No. of Eyes)	P Value ^a
60 degrees, superior temporal side of the optic disc	23.0 ± 4.8 (12 eyes)	25.5 ± 4.9 (21 eyes)	.174
60 degrees, inferior temporal side of the optic disc	22.9 ± 3.7 (9 eyes)	27.9 ± 5.8 (21 eyes)	.026

^at test.

appeared thinned but not disrupted.^{6,38,39} Altogether, these results indicate that both lost nerve fiber bundles and nerve fiber bundles with decreased thickness and width can be seen as NFL defects on fundus photography or red-free SLO images.

We further evaluated the mean nerve fiber bundle width in the area without visual field defect and the NFL defect on fundus photography or red-free SLO imaging and found that the nerve fiber bundle width was significantly lower in eyes with glaucoma than in normal eyes on the inferior temporal side of optic disc (60 degrees from the horizontal line). These results suggest that narrowing of nerve fiber bundles on AO SLO may precede the NFL defect on fundus photography. Using OCT, it has been reported that the NFL thickness can be decreased even in areas without visual field defects.^{40,41} Na and associates reported that perimetrically normal hemifields of glaucomatous eyes had significantly lower macular ganglion cell complex and circumpapillary NFL thickness than did the corresponding retinal regions of healthy eyes.⁴⁰ Choi and associates reported abnormal NFL parameters in quadrants without visual field defects in normal-tension glaucoma.⁴¹ Thus, NFL damage, seen as narrow nerve fiber bundles on AO SLO or thin NFL on OCT, may be present before visual field defects and NFL defects are detectable. Further longitudinal studies using AO SLO are needed to confirm this interpretation.

The visual field MDs for each area were correlated with corresponding nerve fiber bundle widths, suggesting that structural abnormalities in the NFL are associated with visual function loss. Many earlier studies have correlated circumpapillary NFL thickness measured using scanning laser polarimetry (GDx; Carl Zeiss Meditec Inc, Dublin, California, USA), confocal scanning laser ophthalmoscopy (Heidelberg Retina Tomograph [HRT]; Heidelberg Engineering, Heidelberg, Germany), and OCT with visual field function.^{42–44} Recent studies using SD OCT have reported correlations between visual field clusters and circumpapillary NFL thickness.^{45–48} However, previous studies have not addressed the relationship between nerve fiber bundle width and visual field index. Although preliminary, our findings indicate that nerve fiber bundle width measured by AO SLO may be objective and quantitative indicators of visual function in eyes with glaucoma.

Our study has several limitations. First, although the lateral resolution of AO SLO is superior to that of commercially available SLO or SD OCT equipment, currently available AO imaging equipment cannot show nerve fiber bundles clearly in eyes with media opacity; these eyes were excluded from this study. Because the number of study subjects was small, we cannot exclude the possibility of selection bias. Second, there is currently no automated segmentation software available for measuring nerve fiber bundle widths; thus, we performed all segmentations manually. However, we previously showed good interobserver repeatability with this technique, and the ICC for interobserver measurements was high in this study.²⁶ Third, images obtained very near the optic disc may show stacks of bundles rather than individual bundles; the thickness of the NFL just near the optic disc is considerably larger, and several bundles may lie on top of one another. However, in the current study, nerve fiber bundle widths were measured along a circle with a diameter of 3.4 mm centered on the optic disc, and histologic studies have shown that most nerve fiber bundles are separated at this distance.⁸ Further studies are needed to investigate the optimal distance for assessment of nerve fiber bundle width in detecting and monitoring glaucoma.

In conclusion, our study demonstrates that AO SLO imaging allows visualization of individual nerve fiber bundles and measurement of their width, which has not been possible using current glaucoma imaging devices. Our results suggest that: (1) nerve fiber bundle width may change in proportion to its thickness in eyes with glaucoma as compared with controls; (2) NFL defects seen on fundus photography or red-free SLO imaging may not be actual nerve fiber defects, but rather indications of nerve fiber bundle narrowing; (3) narrowing of the nerve fiber bundles on AO SLO may exist before the visual field defect; and (4) changes in nerve fiber bundles seen on AO SLO images correlate with functional loss. Our results suggest that nerve fiber bundle imaging with AO SLO is a useful tool for

detecting and quantifying nerve fiber bundle abnormalities and for assessing their association with visual field changes in eyes with glaucoma. We hope to perform longitudinal

studies using AO SLO to learn more about the involvement of this peculiar feature in the pathogenesis of glaucoma, for better management of this disease.

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SUPPLEMENTAL MATERIAL

The adaptive optics (AO) subsystem contains a liquid-crystal-on-silicon spatial light modulator (LCOS SLM; Hamamatsu Photonics, Hamakita, Japan), a Shack-Hartmann wavefront sensor, and software. The light source for wavefront sensing is a 780-nm laser diode (the light power is 70 μ W at the subject's pupil). Custom software controls the liquid-crystal spatial-light modulator and the wavefront sensor to reduce the residual wavefront aberrations arising from the AO scanning laser ophthalmoscopy (SLO) system and the subject's eye. The LCOS SLM consists of a parallel-aligned liquid crystal layer, a multilayer dielectric mirror, and active-matrix circuits with pixilated electrodes.¹ The number of pixels is 792×600 and the pixel size is $20 \times 20 \mu\text{m}$. The multilayer dielectric mirror was designed to have 99% reflectivity in the wavelength range of the laser diode laser and the SLO. Although the stroke of the LCOS SLM is nearly 1 wavelength, an effective phase stroke of 20 wavelengths or more can be achieved using the phase-wrapping technique.² The wavefront sensor consists of a lens array and a high-speed camera.³ The lens array has 25×25 square lenslets in a 10×10 -mm active sensor area. The software performs closed-loop AO control at a rate of 10 Hz. Aberration sensing and correction were performed within a circular area. The diameter of the area at the corneal plane was approximately 5.5 mm, and the number of lenslets in this area was approximately 225.

The SLO subsystem uses an 840-nm superluminescent diode with 50-nm full width and the illuminating source at half maximum (the light power at the subject's pupil is 210 μ W). The custom computer software reads the output of an avalanche photodiode detector synchronized with both the horizontal raster scans by a resonant scanner (SC-30; Electro-Optical Products Corp., Ridgewood, New York, USA) and the vertical scans by a galvano scanner (6230H; Cambridge Technology, Lexington, Massachusetts, USA) to achieve an image acquisition rate of 50 frames per second (each image is 512×320 pixels and covers an area of 3.0×1.9 degrees in width

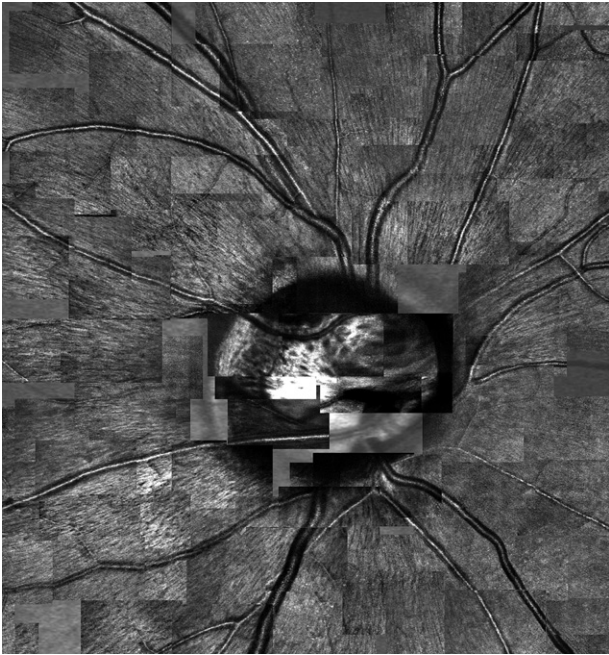
and height, respectively) using both the forward and return sweeps of the resonant scanner.⁴

This subsystem is designed optically to cancel intrinsic aberrations. The defocusing aspect of the aberrations of the entire eye is corrected manually with a Badal optics unit mounted on the translation stage; other aberrations are compensated for by the AO system, which conducts diffraction-limited projection of the fiber tip of the light source onto an arbitrary layer in the retina. Although the LCOS SLM of the AO subsystem in principle functions at only 1 specific wavelength, the authors have confirmed experimentally significant improvements in lateral resolution and image contrast.

The principle of line-scan SLO was used as a wide-field imaging subsystem in which a 910-nm superluminescent diode was used as a light source and a 1-dimensional charge-coupled device was used as a detector and confocal slit, which suppresses scattering of the reflection from the retina. The image acquisition rate is 50 frames per second and the angular field of view is 28 degrees and 24 degrees along the horizontal and vertical directions, respectively, across which the retinal region can be shifted arbitrarily. The AO SLO system is confocal, allowing us to create high-contrast en face images for any plane in the living retina.

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SUPPLEMENTAL FIGURE. Wide-field montage of adaptive optics scanning laser ophthalmoscopy (AO SLO) images using a 3.0×1.9 -degree field of view.



Biosketch

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