RefMoB, a Reflectivity Feature Model-Based Automated Method for Measuring Four Outer Retinal Hyperreflective Bands in Optical Coherence Tomography

Douglas H. Ross,^{1,2} Mark E. Clark,³ Pooja Godara,³ Carrie Huisinigh,³ Gerald McGwin,^{3,4} Cynthia Owsley,³ Katie M. Litts,^{3,5} Richard F. Spaide,⁶ Kenneth R. Sloan,^{1,3} and Christine A. Curcio³

¹Department of Computer and Information Sciences, University of Alabama at Birmingham, Birmingham, Alabama, United States

²Department of Mechanical Engineering, University of Alabama at Birmingham, Birmingham, Alabama, United States

³Department of Ophthalmology, University of Alabama at Birmingham, Birmingham, Alabama, United States

⁴Department of Epidemiology, University of Alabama at Birmingham, Birmingham, Alabama, United States

⁵Vision Science Graduate Program, University of Alabama at Birmingham, Birmingham, Alabama, United States

⁶Vitreous Retina Macula Consultants of New York, New York, New York, United States

Correspondence: Christine A. Curcio, Department of Ophthalmology, University of Alabama at Birmingham, 1670 University Boulevard, VH 360, Birmingham, AL 35294, USA; curcio@uab.edu.

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PURPOSE. To validate a model-driven method (RefMoB) of automatically describing the four outer retinal hyperreflective bands revealed by spectral-domain optical coherence tomography (SDOCT), for comparison with histology of normal macula; to report thickness and position of bands, particularly band 2 (ellipsoid zone [EZ], commonly called IS/OS).

METHODS. Foveal and superior perifoveal scans of seven SDOCT volumes of five individuals aged 28 to 69 years with healthy maculas were used (seven eyes for validation, five eyes for measurement). RefMoB determines band thickness and position by a multistage procedure that models reflectivities as a summation of Gaussians. Band thickness and positions were compared with those obtained by manual evaluators for the same scans, and compared with an independent published histological dataset.

RESULTS. Agreement among manual evaluators was moderate. Relative to manual evaluation, RefMoB reported reduced thickness and vertical shifts in band positions in a band-specific manner for both simulated and empirical data. In foveal and perifoveal scans, band 1 was thick relative to the anatomical external limiting membrane, band 2 aligned with the outer one-third of the anatomical IS ellipsoid, and band 3 (IZ, interdigitation of retinal pigment epithelium and photoreceptors) was cleanly delineated.

CONCLUSIONS. RefMoB is suitable for automatic description of the location and thickness of the four outer retinal hyperreflective bands. Initial results suggest that band 2 aligns with the outer ellipsoid, thus supporting its recent designation as EZ. Automated and objective delineation of band 3 will help investigations of structural biomarkers of dark-adaptation changes in aging.

Keywords: optical coherence tomography, reflectivity, segmentation, retina, ellipsoid, interdigitation, photoreceptors, retinal pigment epithelium, age-related macular degeneration

Spectral-domain optical coherence tomography (SDOCT) is an interferometry technique that uses low coherence light to achieve depth-resolved, comprehensive, and noninvasive cross-sectional views of chorioretinal structure in vivo.¹ The four outer retinal hyperreflective bands of SDOCT (Fig. 1A) are enabled by the precise horizontal alignment of cells (photoreceptors, Müller cells, RPE) and an extracellular matrix that serves as RPE substrate and a vessel wall (Bruch's membrane [BrM]). Band names have been proposed by an international nomenclature consortium² (from inner to outer) as the external limiting membrane (ELM; band 1), ellipsoid zone (EZ; band 2), interdigitation zone (IZ; band 3), and RPE-BrM complex (band 4). These bands invite a subcellular explanation for reflectivity, because the internal structures in the contributing cells are strictly segregated in the vertical axis. This feature, best documented for the photoreceptors³ is also true for the RPE.⁴

The subcellular basis of SDOCT reflectivity can be approached by comparing thicknesses and vertical positions of reflective bands to chorioretinal layers measured histologically. We previously generated from a century of literature an anatomical model of outer retina in normal macula.⁵ By assuming that the ELM and RPE-BrM bands were correctly designated, we found that band 2 closely aligned with the IS ellipsoid (ISel), a conclusion supported by direct imaging-histology correlations in laboratory animals.⁶ Whether band 2 overlaps with the ISel is of interest, because the ISel is packed with mitochondria,⁷ an organelle known for light-scattering⁸ and energy-generating properties.^{9,10} Further, we proposed that band 3 corresponded to the interface of outer segment (OS) tips with RPE apical processes (microvilli).⁵ These fine processes, up to 15 μ m long, form a candelabra-like arrangement with cone OS, which are shorter than rod OS. Other

FIGURE 1. Approaches to segmenting the four outer retinal hyperreflective bands. (A) SDOCT B-scan of the macula of a normal human retina, generated using a Spectralis (Heidelberg Engineering) and the manufacturer's standard contrast adjustment for display. Reflectivity amplitude is indicated by intensity with white indicating higher reflectivity. (B–D) are enlargements of the *yellow-bounded area* in (A). (B) Enlargement with no segmentation information. (C) Segmentation scheme based on minimum amplitude to separate regions. *Cyan curves* at minimum values separate contiguous regions I, II, III, and IV. (D) Segmentation of hyperreflective band as features. Three curves represent the inner boundary, centerline, and outer boundary of each band. The distance from the inner to outer boundary is band thickness, which is then compared against the vertical height of retinal features in histology.

investigators call band 3 the posterior tips of OS,¹¹ external tips of photoreceptors,¹² and cone and rod OS tips, where this band bends inward from the RPE in the near periphery.¹³

The purpose of this study was to validate an automated reflectivity model-based (RefMoB) method that determines thicknesses and vertical positions of the four outer hyperreflective bands in normal maculas. Segmentation methods for SDOCT scans commonly create boundaries based on signal amplitude, difference, or texture. Regions are the spaces between boundaries (Fig. 1C). RefMoB models band appearance as the sum of four Gaussians and describes the position and thickness of each band as a centerline and a width. It does not depend on finding edges of features in images (Fig. 1D). Previous SDOCT segmentation methods for the four outer bands have reported one,^{14–16} two (Tolliver D, et al. *IOVS* 2008;49:ARVO E-Abstract 6017 and Refs. 17–19, 20–24), three,^{25–28} four,^{29–33} five,^{13,34} and six^{35,36} boundaries or centerlines. RefMoB finds eight features associated with four bands (Fig. 1D). A secondary goal is to compare these band thicknesses and positions with the published anatomical model⁵ and histological inner segment (IS) lengths in normal human eyes.³⁷

METHODS

RefMoB uses a multistage process, starting with crude estimates based on an anatomical model and intensity values in B-scans. Information gleaned at every stage is evaluated for reliability. Reliable data are propagated to nearby locations, providing two-dimensional context. Finally, we model the reflective response of each band as a Gaussian. The Gaussians overlap, sometimes to the point of appearing to merge. An optimization process, guided by anatomical constraints, is used to separate out descriptions of all four bands. This removes a systematic shift in edges caused by overlap and enables

separation of individual bands.³⁸ This is particularly important for band 3.

A Gaussian can be fully described by two parameters: μ and σ . We report position as μ and thickness as twice σ . This is the same as the distance between the maximum gradient on either side of a perfect Gaussian. We do not actually locate maximum gradients in the final stage; we do use gradient information in earlier stages. This measure of thickness is not equivalent to the full width at half maximum, although conversion from one to the other is straightforward for perfect Gaussians. For comparison with anatomy, the thickness estimates were corrected for the point spread function (PSF) of the SDOCT instrument, as described.⁵

Linear reflectivities are used in RefMoB. Results obtained with RefMoB were compared with those generated by human evaluators using two manual methods that both used contrast-adjusted images identical to those produced by a Spectralis (Heidelberg Engineering, Heidelberg, Germany), at the request of the evaluators. This adjustment generates systematic overestimates in width.⁵ In addition, the combination of overlap between reflective responses³⁸ and contrast adjustment will cause band positions to shift slightly, as illustrated in a simulation (Figs. 2B, 2D). Bands 3 and 4 shift inward and outward, respectively.

Implementation Details

The RefMoB proceeds in four stages. Each stage refines the model produced by the previous stage and evaluates the quality of results before passing them on (“islands of reliability”). The stages are as follows: (1) find the retinal region in B-scans, using a model of the imaging process; (2) estimate center position and thickness for bands 2 to 4, using standard image processing techniques; (3) fit a summation of Gaussians to bands 2 to 4; and (4) process band 1 (band 1 is

FIGURE 2. A simulation predicts that positions of reflective bands as well as thickness are affected by contrast adjustment for display. Thickness of all bands increases. Position of band 3 moves inward and band 4 moves outward. Band 1 and 2 positions move, but the displacement is not visible at this scale. (A) Raw reflective Gaussian responses based on an A-scan (orange, red, green, and blue curves). (C) Contrast-adjusted summation of Gaussians (cyan curve). Points of maximum gradient for each band are indicated by color-coordinated triangles. The need for contrast adjustment for human evaluation is clear comparing raw data (B) with contrast-adjusted data (D). The band 1 Gaussian amplitude has been increased by a factor of 10 to make it visible for display only.

much dimmer than the other three bands). The RefMoB was written in Java by using the ImageJ environment (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The ImageJ plugin is available on request.

1. Boundaries Process. Retinal boundaries define the overall region of interest. Three progressively blurred images at full resolution are generated. The retina is located in the most blurred image and refined in stages. Top and bottom boundaries are based on maximum gradient (Fig. 3).

2. Initial Parameters Process. The Gaussians associated with each band are characterized by amplitude, μ , and σ . For every A-scan, there are, three substeps. Substeps 2 and 3 are iterated 10 times:

Substep 1 finds a three-peak pattern representing bands 2 to 4. The pattern must meet threshold requirements for peak amplitudes and distances between peaks (Figs. 4A, 4B) (see Supplementary Material and Ref. 39 for values). Patterns are further qualified by removing outliers via a moving window average (Fig. 4C). Results are reported only for A-scans that show a clear, reliable pattern.

Substep 2 propagates band center position to neighboring A-scans. Reliable A-scan data are connected with a polyline. For each A-scan, this position is refined by moving toward the local maximum. The polyline is then smoothed (Fig. 4D, center lines).

Substep 3 propagates width information to neighboring A-scans. Boundary polylines are created as offsets (based on expectations derived from the anatomical model) from band centers. At each A-scan, boundaries are refined by moving toward the local maximum gradient and smoothed as in substep 2 (Fig. 4D, boundary lines).

FIGURE 3. Finding the initial retinal boundaries by finding gradients on successively less-filtered B-scan images. The unfiltered B-scan is in (D), and (C) to (A) are progressively more filtered. (A) Yellow curves are the initial boundaries based on the maximum values of the two features created by Gaussian filtering. (B–D) Maximum positive and negative gradient boundary curves are found on more to less filtered images with the final curves on the unfiltered B-scan (D). Curves from more filtered images are used as initial locations, for an iterative method, to find the maximum gradients in the next less filtered image. The use of a gradient method on successively less filtered B-scans removes the requirement for a threshold value to find the retinal boundaries.

3. Gaussian Fitting Process Bands 2 to 4. Starting with “well-understood” A-scans from Step 1, Step 2 produced estimates guided by a blend of local image data and information propagated from neighboring A-scans. The output of Step 2 is a center position and upper and lower boundaries for each band, at every A-scan. In Step 3, every A-scan is subjected to an optimization process that describes each A-scan as the sum of three Gaussians, subject to constraints imposed by anatomical models and results from neighboring A-scans.

For each of three Gaussians, the center is initialized to the band center position and σ is set to half the difference between inner and outer boundaries from Step 2. The initial amplitude is set to the A-scan amplitude at the center position. The method of Brent⁴⁰ as implemented by Lau⁴¹ is used for optimization. The sum of algebraic error squared is minimized, and results are smoothed (Figs. 5A, 5B).

Optimization methods implement model-based constraints to prevent unreasonable results by biasing the error term. For constraints we used positive amplitude, plausible thicknesses, limited center movement from the initial value, and non-overlap of bands (see Supplementary Material). These constraints primarily affect A-scans with two local maximum values representing three underlying structures (Fig. 5B).

4. Gaussian Fitting Process Band 1. The amplitude of band 1 is significantly smaller than bands 2 to 4 (Fig. 6) and requires special handling. Band 1 center is found initially as the first peak above band 2. The signal contribution from bands 2 to 4 is subtracted from the raw intensities before describing band 1.

FIGURE 4. Finding the initial center and thickness values for the Gaussian fitting. (A, B) Determining the A-scans, with a high confidence of three band peaks being present. Initial choices for peak locations are based on peak absolute amplitudes, a ratio of the amplitudes, and the distance between peaks. (C) Outliers are eliminated based on a threshold with respect to a moving window average, leaving the red, green, and blue peak locations. (D) The center curve and the inner and outer band boundary curves are created by iterative refinement using a region and edge criterion respectively.

Validation of RefMoB

Seven SDOCT volumes (Spectralis; Heidelberg Engineering) from seven eyes of five individuals with normal retinas aged 28 to 69 years were used for comparing manual and automated measurements: three volumes from three individuals from a private retina referral practice (author RFS) and four volumes from two individuals in normal macular health enrolled in the Alabama Study on Early Age-Related Macular Degeneration⁴² (author CO). One eye was used from each individual for measuring band thickness and position. For both purposes, one scan through the foveal center and another at 2 mm superior to the fovea (perifovea, near the rod peak⁴³) were chosen from each volume. Resolution along an A-scan for these

files is approximately 4 μm per pixel and between A-scans is approximately 6 μm per pixel.

Scans were evaluated by RefMoB and two manual segmentation methods with different user interfaces. One manual method delineated boundaries by placing interpolation points for spline curves, adapted from SDOCT studies of optic nerve head.⁴⁴ Another method involved placing points at band boundaries at predefined eccentricities, adapted from histological and OCT studies of macula.^{37,45,46} Each manual method was performed on each of the 14 B-scans by three masked evaluators working independently. Two evaluators (MEC and PG) were experienced with SDOCT. Manual evaluations using the point-based method, which was faster to use, were compared with each other and to RefMoB using intraclass correlations (ICCs). Reliability indicates by ICC values greater than 0.75 is excellent, between 0.4 and 0.75 is fair to good, and less than 0.4 is poor reliability. Descriptive statistics for bands included coefficient of variation (CV), equal to standard deviation divided by mean.

This research complied with the tenets of the Declaration of Helsinki and was approved by the institutional review boards of University of Alabama at Birmingham and Vitreous Retina Macula. Informed consent was obtained from all participants.

Histology

We previously measured layer thicknesses in macula-wide, sub-micrometer sections through the foveas of 18 human eyes preserved at less than 6 hours postmortem.³⁷ At standard locations, IS of cones displaying a continuity of cell body, IS, and OS ("picket fence" configuration) were measured. When viewed with a $\times 60$ 1.4 NA oil immersion objective (Olympus, Central Valley, PA, USA), cone IS myoid (ISmy) was pale staining, with fine deep blue streaks attributable to embracing Müller cell microvilli, and ISel was overall deep staining, with individual longitudinal mitochondria visible in the best preserved specimens.^{47,48} Because distances between the IS and RPE are affected by postmortem detachment of OS and compaction of RPE apical processes, only IS lengths and positions were compared with SDOCT band delineation results.

RESULTS

Intraclass correlations among manual evaluators for band position in foveal and perifoveal scans were fair to good

FIGURE 5. Fitting the summation of three Gaussian functions. (A) Summation of three Gaussian functions fit to three local maximum values. (B) Summation of three Gaussian functions fit to two local maximum values. The band 2 to 4 Gaussian fit function curves are red, green, and blue. The image data are blue crosses. The summation of the three Gaussian functions is a black curve.

FIGURE 6. Amplitude of band 1 is much smaller than amplitudes of bands 2 to 4. The *inset* shows the reflectance amplitude of band 1 in the range of hundredths where the other bands amplitudes are expressed in tenths. The difference in amplitude requires separate processing of band 1 data. The maximum amplitudes are of the same order when contrast adjusted. See Figures 2 and 8 for contrast-adjusted data.

(0.310–0.700, Table 1, column 4, bottom). Intraclass correlations among evaluators for band thickness were poor (0.035–0.490, Table 1, column 4, top). Intraclass correlation between manual evaluators and RefMoB for band position was also fair to good (0.245–0.777, Table 1, columns 7–10 bottom). In all but a few locations, ICCs between evaluators and RefMoB were poor for band thickness (0.282–0.214, Table 1, columns 7–10 top), due to RefMoB reporting systematically thinner bands, as expected.

Band boundaries relative to band 1 centerline from manual evaluators and RefMoB were compared graphically (Fig. 7). Band shape in Figure 7 differs from that in the original SDOCT scans because of normalization of position to band 1. In fovea and perifovea, overall shapes of manually segmented reflective bands agree with those defined by RefMoB. Relative to RefMoB-segmented bands, manually segmented bands 1, 2, and 4 are thicker, and the center and outer boundary of band 4 is shifted outward.

RefMoB results for band thickness and position relative to band 1 (Tables 2, 3) prompt several observations. First, variability in both thickness and vertical position are location-dependent. Coefficient of variation for thickness is less than or equal to 0.7 and less than or equal to 0.3 in foveal and perifoveal scans, respectively. Coefficient of variation for position is overall less, at less than or equal to 0.13 and less than or equal to 0.10, respectively. Using band 1 as a reference causes CV for position to increase from band 2 to band 4. Coefficient of variation for thickness is greatest at low eccentricities of foveal B-scans. Second, with respect to absolute band thicknesses, band 1 ranges over 5.1 to 8.9 μm in the foveal scan and 5.7 to 7.4 in the perifoveal scan. Band 2 is 11.6 μm (SD 5.8) at the foveal center, 6.0 to 6.1 μm (SD 0.5–0.7) at ± 1500 μm eccentricity in the foveal scan, and 5.9 to 6.5 μm across the perifoveal scan. Band 3 is also quite thick at 11.3 μm (SD 2.9) in the foveal center with a range of 9.0 to 10.5 μm across the perifovea. Band 4 is 12.8 to 14.5 μm across the foveal scan and 13.6 to 14.5 μm across the perifoveal scan. Third, band 1 deviates inward in the foveal center, where the distance between band 1 and 4 is 90.6 μm (SD 3.3), then

TABLE 1. Average ICC by Method, Location, and Band

1	2	3	4	5	6	7	8	9	10
B-scan	Measure	Band	Mean ICC*	ICC*,†-‡	ICC*,†-§	ICC*,‡-§	ICC*, -†	ICC*, -‡	ICC*, -§
Fovea	Thickness	1	0.490	0.545	0.433	0.506	0.166	0.193	0.214
Fovea	Thickness	2	0.270	0.251	0.313	0.267	0.107	0.127	0.071
Fovea	Thickness	3	0.320	0.356	0.208	0.420	−0.010	0.042	0.032
Fovea	Thickness	4	0.085	0.041	0.181	0.078	0.049	0.000	0.001
Perifovea	Thickness	1	0.160	0.347	0.099	0.099	−0.030	−0.038	−0.021
Perifovea	Thickness	2	0.190	0.105	0.287	0.028	0.003	0.010	−0.002
Perifovea	Thickness	3	0.035	−0.069	0.070	0.082	0.101	−0.107	−0.282
Perifovea	Thickness	4	0.250	0.277	0.216	0.212	0.052	0.045	0.080
Fovea	Position	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fovea	Position	2	0.430	0.474	0.343	0.488	0.526	0.652	0.496
Fovea	Position	3	0.580	0.570	0.466	0.703	0.511	0.687	0.531
Fovea	Position	4	0.500	0.571	0.405	0.542	0.245	0.461	0.261
Perifovea	Position	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Perifovea	Position	2	0.310	0.403	0.161	0.356	0.500	0.698	0.266
Perifovea	Position	3	0.690	0.697	0.607	0.705	0.683	0.793	0.642
Perifovea	Position	4	0.700	0.781	0.614	0.665	0.582	0.777	0.432

* Intraclass correlations (Shrout-Fleiss reliability: random set) were calculated at the eccentricity level using the SAS (SAS, Inc., Cary, NC) procedure “Proc Mixed” to calculate the average ICC at the band level and account for individual (subject)-level clustering.

† Evaluator 1.

‡ Evaluator 2.

§ Evaluator 3.

|| RefMoB.

FIGURE 7. Boundaries of outer retinal hyperreflective bands determined using manual and RefMoB methods on seven scans of five healthy human subjects. (A) Boundaries determined from B-scans through superior perifovea. (B) Boundaries determined from B-scans through fovea. The pairs of colored curves mark the inner and outer boundaries of each band plotted relative to the band 1 centerline. Each curve is the average of all the measurements for that location on all B-scans. The light color surrounding the band average is the SD. The manual SD is the measurement compared with the average of the three evaluators. The RefMoB SD is also compared with the three evaluators normalized to the RefMoB average.

decreases to 69.2 and 68.0 μm (SD 2.3, 2.9) at 1500 μm . This deviation is still apparent at zero eccentricity in the superior perifovea (Fig. 7A).

The RefMoB position and thickness for bands 2 and 3 at the foveal center and 1.5 mm eccentricity were compared with those predicted by our anatomic model (Fig. 8).⁵ Band 2 aligns with the outer half of the ISel, band 3 aligns with the interdigitation of cone OS and RPE apical processes, and band 1 aligns with the ELM (Fig. 8). Band 2 thickness and vertical

position were also compared with mean length of cone ISel in fovea and perifovea of 18 normal maculas (Fig. 9). These thicknesses are slightly larger than the anatomical model, and they decrease with distance from the foveal center. Band 2 overlies the outer one-third of the measured ISel. Because processing-related tissue volume change could reduce histologic IS lengths, we checked the robustness of fits with shrinkage estimates of 5% to 15% linear. In none of these comparisons did band 2 overlap the boundary between IS and

TABLE 2. Thickness of Reflective Bands as Determined by RefMoB

B-scan	Data	Band	Eccentricity														
			−1500	−1000	−600	−400	−200	−100	−50	0	50	100	200	400	600	1000	1500
Fovea	Mean	1	7.2	8.2	8.1	8.3	7.4	6.5	5.5	5.1	6.5	7.4	8.3	8.9	8.8	8.3	6.9
		2	6.0	6.1	6.4	6.7	8.4	10.6	11.2	11.6	10.8	10.5	8.6	7.2	6.5	6.2	6.1
		3	10.3	8.1	7.6	7.9	8.7	10.2	11.1	11.3	10.0	9.5	8.2	7.8	8.1	9.5	9.5
		4	14.5	14.4	14.4	13.8	13.2	13.9	13.3	13.4	12.8	13.2	13.3	13.8	13.2	13.0	13.3
	SD	1	0.7	1.0	1.3	2.1	1.1	2.8	2.5	3.4	3.4	2.6	2.2	1.3	1.4	1.2	1.1
		2	0.5	0.8	1.1	1.3	2.4	4.3	5.1	5.8	6.1	5.5	3.8	1.5	1.0	0.6	0.7
		3	2.1	1.1	1.1	1.3	0.8	2.9	3.0	2.9	2.8	2.7	1.5	0.7	1.0	2.9	1.8
		4	2.0	1.8	1.6	1.0	1.2	1.3	2.0	1.7	1.9	1.2	1.4	1.4	1.9	1.0	1.3
	CV	1	0.09	0.12	0.16	0.26	0.15	0.44	0.46	0.66	0.51	0.35	0.26	0.15	0.16	0.15	0.15
		2	0.07	0.13	0.17	0.19	0.28	0.40	0.45	0.50	0.56	0.53	0.45	0.21	0.16	0.10	0.11
		3	0.21	0.14	0.14	0.16	0.09	0.28	0.27	0.26	0.28	0.29	0.18	0.09	0.13	0.30	0.18
		4	0.14	0.13	0.11	0.07	0.09	0.09	0.15	0.13	0.15	0.09	0.11	0.10	0.14	0.08	0.10
Perifovea	Mean	1	7.4	6.1	6.2	6.1	6.4	6.5	6.8	6.8	6.7	6.6	6.1	5.7	6.2	6.6	6.2
		2	6.0	6.1	6.4	6.2	6.5	6.5	6.4	6.4	6.4	6.3	6.3	6.2	6.1	5.9	6.3
		3	9.0	9.8	9.5	10.1	9.8	10.0	10.1	10.0	9.8	9.2	10.0	10.3	10.3	9.4	10.5
		4	14.2	14.5	14.3	14.5	13.8	13.9	14.1	14.1	14.0	13.6	13.7	13.6	13.7	14.1	13.4
	SD	1	1.2	0.6	1.6	0.6	1.2	0.7	0.7	0.7	0.8	0.8	1.0	1.3	1.3	1.3	1.3
		2	0.5	0.9	1.0	1.0	1.1	0.8	0.8	0.8	0.8	0.8	0.8	0.7	0.7	0.6	0.9
		3	1.5	1.7	2.5	3.4	2.5	2.5	2.2	2.2	1.9	1.4	2.5	2.8	3.4	2.6	3.1
		4	2.2	1.6	1.7	1.0	1.1	1.1	1.4	1.3	1.3	1.3	1.2	1.5	1.1	1.5	1.7
	CV	1	0.17	0.10	0.26	0.10	0.18	0.10	0.10	0.10	0.11	0.11	0.16	0.23	0.20	0.20	0.20
		2	0.08	0.14	0.15	0.15	0.16	0.13	0.13	0.13	0.13	0.13	0.13	0.11	0.12	0.11	0.15
		3	0.16	0.18	0.27	0.34	0.25	0.25	0.22	0.22	0.19	0.16	0.25	0.27	0.32	0.27	0.29
		4	0.16	0.11	0.12	0.07	0.08	0.08	0.10	0.09	0.09	0.10	0.09	0.11	0.08	0.11	0.13

The mean and SD in micrometers and CV are from five normal adult eyes from five individuals. The thickness values are determined from Gaussian sigma values with a 7- μm PSF for an infinitely thin reflector removed.

TABLE 3. Distance of Band 2 to 4 Centers From Band 1 Center, Determined by RefMoB

			Eccentricity														
B-scan	Data	Band	−1500	−1000	−600	−400	−200	−100	−50	0	50	100	200	400	600	1000	1500
Fovea	Mean	2	23.7	25.5	28.4	30.5	31.5	32.6	32.3	32.8	32.2	31.7	30.7	30.3	28.5	26.6	24.4
		3	47.9	52.2	58.0	63.2	68.8	73.2	73.8	74.1	72.3	70.7	67.6	62.3	57.8	53.2	47.4
		4	68.0	71.8	78.1	81.6	85.5	88.8	90.0	90.6	89.1	87.5	84.8	81.0	77.4	72.9	69.2
	SD	2	1.3	1.0	1.2	1.9	0.8	3.5	4.0	4.2	3.5	2.2	1.5	1.4	1.6	1.0	0.8
		3	2.0	1.5	1.8	2.6	2.9	3.2	3.9	4.2	4.0	2.5	1.6	1.5	2.5	1.3	1.1
		4	2.9	3.2	2.7	3.2	3.4	2.8	2.8	3.3	3.4	2.8	2.7	2.6	3.3	2.4	2.3
	CV	2	0.05	0.04	0.04	0.06	0.03	0.11	0.12	0.13	0.11	0.07	0.05	0.05	0.06	0.04	0.03
		3	0.04	0.03	0.03	0.04	0.04	0.04	0.05	0.06	0.06	0.04	0.02	0.02	0.04	0.03	0.02
		4	0.04	0.04	0.03	0.04	0.04	0.03	0.03	0.04	0.04	0.03	0.03	0.03	0.04	0.03	0.03
Perifovea	Mean	2	23.6	23.7	23.6	24.5	24.4	24.8	24.8	25.0	25.0	25.0	24.6	24.4	24.1	23.5	22.8
		3	44.3	45.1	45.4	47.3	48.1	48.7	48.5	49.0	49.2	49.0	49.0	47.9	47.5	44.8	43.2
		4	66.9	67.6	67.6	68.9	69.7	70.3	70.3	70.2	70.0	69.6	69.3	68.8	67.9	66.4	66.0
	SD	2	0.8	0.7	1.1	1.1	1.5	1.8	1.9	1.7	1.5	1.5	1.4	1.2	1.2	1.0	0.5
		3	1.3	1.2	3.2	3.8	4.2	4.7	4.8	4.9	4.9	4.3	5.0	3.6	4.0	3.0	1.0
		4	2.4	2.7	3.8	4.6	5.2	5.4	5.2	4.9	4.7	4.3	4.6	3.8	3.5	3.6	2.1
	CV	2	0.04	0.03	0.05	0.04	0.06	0.07	0.08	0.07	0.06	0.06	0.06	0.05	0.05	0.04	0.02
		3	0.03	0.03	0.07	0.08	0.09	0.10	0.10	0.10	0.10	0.09	0.10	0.07	0.08	0.07	0.02
		4	0.04	0.04	0.06	0.07	0.07	0.08	0.07	0.07	0.07	0.06	0.07	0.06	0.05	0.05	0.03

The mean and SD in micrometers and CV are from five normal adult eyes of five individuals. Band 1 distance is not shown because all the values would be zero, as that was the reference.

OS. All measurements are derived from an ideal, continuous model fit to the data; the resolution of the images themselves is approximately 5 μm per pixel.

DISCUSSION

Our principal goal was validating RefMoB, a fully automatic method for describing the four outer retinal hyperreflective bands of SDOCT as overlapping Gaussian shapes. A secondary goal was to provide accurate and objective initial thicknesses and positions of these bands across normal macula for comparison with normal anatomy. As revealed theoretically and empirically (Figs. 2, 7), RefMoB's use of linear reflectivities

not only provided thinner bands than contrast-enhanced reflectivities but also shifted the position of band 4 inward relative to the ELM. In this dataset, we find that band 2 aligns with the outer one-third of the histological ISel, band 1 is surprisingly thick relative to the anatomical ELM, and band 3 is cleanly delineated from band 4, even in the fovea.

Automated methods that generate five or more outer retinal boundaries (Table 4) all detect the inner and outer boundaries of band 2. The RefMoB and one other method find band 1 boundaries. Only RefMoB determines both boundaries of bands 3 and 4. The method of Srinivasan et al.¹³ works primarily in one dimension on an A-scan. Ehnes et al.³⁶ use an optimal path in two dimensions. Quellec et al.³⁴ use a three-dimensional graph search. Kafieh et al.³⁵ used diffusion maps

FIGURE 8. Average band thickness values are compared with an anatomical model.⁵ Band 1 was aligned with the ELM and band 4 with the RPE-Bruch's complex. Heights of the colored bars indicate band thickness based on maximum gradient of the dashed line Gaussians. The solid curves are average Gaussian functions fit to raw reflectivities. The dashed line Gaussians have the 7- μm PSF for an infinitely thin reflector removed. The height of band 1 has been increased by a factor of 10 to make it visible. (A) Average of fovea data from Tables 2 and 3 for eccentricities from -100 to $100 \mu\text{m}$. (B) Average of perifovea data from Tables 2 and 3 for eccentricities from -1500 to $1500 \mu\text{m}$

FIGURE 9. Band 2 overlies the outer one-third of the histological ISel. Band thicknesses are from Table 2. *Solid lines* indicate inner border of cone ISel (green) and OS (blue) at these eccentricities in histological sections of 18 normal maculas of donors age 40 to 92 years.³⁷ *Symbols* indicate these borders according to the anatomical model shown in Figure 8.⁵ Band 2 vertical positions are expressed relative to band 1, the position of which was secure in histologic specimens despite retinal detachment due to postmortem delay to fixation.

in two and three dimensions. All four methods use edge gradients to determine boundaries, with Kafieh et al.³⁵ using edges in the spectral domain for band 2 to 4 boundaries and texture for band 1 center. The RefMoB and one other method¹³ report using linear reflectivities; other methods do not specify. The RefMoB uniquely accounts for interaction between bands,³⁸ allowing separation even when one reflective response is in the wing⁴⁹ of another (Fig. 5B). The fitting process filters by including multiple data points along an A-scan. Finally, the crude-to-refined process propagates information from “islands of reliability.” Repeated smoothing reduces variability across nearby A-scans, removing outliers but possibly also masking natural variability.

We compared the results of RefMoB for linear reflectivities with the results of human evaluators using contrast-adjusted images, finding that agreement among evaluators was moderate, and better for position than for thickness. Evaluators differed in identifying top and bottom boundaries but placed boundaries consistently with respect to the band centerline. Band position is based on the average of boundary evaluations, and thickness is based on differences. Although an evaluator who placed the maximum gradient at a lower intensity would create a wider band than an evaluator who placed the maximum gradient at a higher intensity, the between-evaluator average of positions would be similar. These results underscore the need for objective band measurements using linear reflectivities.

Using RefMoB, we found that band 2 position aligns with the ISel across the central 3 mm of macula, thus extending our original conclusion based on contrast-adjusted reflectivities at two anchor locations. Importantly, the greater accuracy

afforded by RefMoB revealed that band 2 thickness does not correspond with the entire ISel, but rather the outer one-third, near the OS. Our results can be compared with those recently obtained by adaptive optics OCT (AOOCT), a technology that discloses individual photoreceptors as two specular surfaces.⁵⁰ Two AOOCT studies, one with a small sample of cells⁵¹ and one with a large sample,⁵² reported 4.7 to 4.8 μm for the median height of individual reflective features within band 2, greater than a single plane when PSF is accounted for, yet less than the anatomical ISel. The larger study (Jonnal et al.⁵²) also measured peak-to-peak distances of AOOCT reflections, concluding that band 2 lies closer to band 3 than band 1. Comparable measures obtained with RefMoB (Table 5) shows, in contrast, that band 2 is closer to band 1 than band 3. Jonnal et al.⁵² attributed band 2 to an optically rough, slanted surface where progressively wider nascent disks of the OS base appose the tapered IS apex.⁵³ These authors⁵² also suggested that band 2 appears thicker in SDOCT than in AOOCT because of variation in IS height; although not mentioned, the statistical nature of speckle is an additional variability source. Both a thin and thick reflectance can be visualized using rapid line scan OCT at the IS of frog retina,⁶ a complexity reminding us that the refractive index gradients underlying each band's reflectivity are multifactorial. Factors contributing to band 2 include IS taper^{54,55} and the matrix sheath surrounding each photoreceptor.^{56,57} Because mitochondria are strong light scatterers⁵⁸ and occupy approximately 75% of ISel volume,⁷ they are candidate reflectivity sources, influenced by organelle spacing and the metabolic state of processes such as oxidative phosphorylation, calcium buffering, and apoptosis regulation.^{8,59} Recent comparisons of clinical imaging with human eye histopathology^{47,48} and experimental studies with animal models (Li W, et al. *IOVS* 2014;ARVO E-Abstract 2174) revealed marked SDOCT reflectivity changes associated with mitochondrial perturbation, consistent with mitochondria generating reflections in vivo. Until results obtained using SDOCT and AOOCT are reconciled and histological correlates are confirmed, the EZ nomenclature incorporates optically significant features at this vertical level.

RefMoB separates band 3 (IZ) from band 4 in these relatively normal eyes. Multiple subcellular sources contribute to band 3. Melanosome translocation within apical processes is detectable by SDOCT in retinomotor movements of frog retina,⁶⁰ and band 3 is visible even in albinism patients lacking melanin, implicating OS.^{61,62} The IZ visibility is affected by the direction of light entry through the pupil,^{11,63} yet we detected band 3 in all subjects. It is reduced or absent in cone-rod dystrophy⁶⁴ and outer retinal atrophy of AMD.⁶⁵ Apical processes contain abundant retinoid processing proteins critical for vision^{66,67} and are historically difficult to study ex vivo due to their delicacy. Noninvasive monitoring via RefMoB will be informative.

Band 1 is less reflective than other bands, yet at 5- to 8- μm thickness was nearly as thick as band 2. Band 1 is universally

TABLE 4. Comparison of Methods Detection Outer Retinal Boundary in SDOCT

Year	Author	Reference No.	Total Boundaries	Total Outer Retinal Boundaries	Band 1	Band 2	Band 3	Band 4	Band 3–4 Separator
2008	Srinivasan et al.	13	6	5	Center	*	Inner	Outer	
2010	Quellic et al.	34	11	5		*	Inner	Outer	*
2013	Kafieh et al.	35	12	6	Center	*	Inner	Outer	*
2014	Ehnes et al.	36	12	6	*	*	Inner	Outer	
	Ross et al. (current study)		9	8	*	*	*	*	

Methods were included that generated five or more boundaries in the outer retinal region.

* Indicates that the inner and outer boundary were found by the reported method.

TABLE 5. Center-to-Center Distances Among Bands 1, 2, and 3, Determined by RefMoB

B-scan	Data	Bands	Eccentricity															
			−1500	−1000	−600	−400	−200	−100	−50	0	50	100	200	400	600	1000	1500	
Fovea	Mean	1-2	23.7	25.5	28.4	30.5	31.5	32.6	32.3	32.8	32.2	31.7	30.7	30.3	28.5	26.6	24.4	
		2-3	24.3	26.7	29.6	32.8	37.3	40.5	41.5	41.3	40.2	39.0	36.8	32.0	29.3	26.6	23.0	
	SD	1-2	1.3	1.0	1.2	1.9	0.8	3.5	4.0	4.2	3.5	2.2	1.5	1.4	1.6	1.0	0.8	
		2-3	1.5	0.8	1.1	1.6	3.2	4.4	3.7	3.6	2.4	1.9	1.1	0.8	1.0	0.9	1.0	
Perifovea	Mean	1-2	23.6	23.7	23.6	24.5	24.4	24.8	24.8	25.0	25.0	25.0	24.6	24.4	24.1	23.5	22.8	
		2-3	20.7	21.4	21.8	22.8	23.7	23.9	23.7	23.9	24.2	24.0	24.4	23.5	23.4	21.3	20.3	
	SD	1-2	0.8	0.7	1.1	1.1	1.5	1.8	1.9	1.7	1.5	1.5	1.4	1.2	1.2	1.0	0.5	
		2-3	1.0	0.9	2.1	3.2	3.2	3.3	3.2	3.6	3.7	3.0	4.0	2.7	3.2	2.2	1.3	

Band position data from five normal eyes, reported in Table 3, was used to compute distances in the manner of Jonnal et al.⁵²

attributed to the ELM, which is less than 0.8- μ m thick.^{7,68} A network of continuous heterotypic adherens junctions formed by photoreceptors and Müller cells, the ELM also contains occludins and other proteins conferring barrier function.⁶⁸ Actin, myosin, and α -actinin aggregate asymmetrically in electron-dense plaques, primarily on the Müller cell side of each junction.⁶⁹ The reduced reflectivity of band 1 in foveola is consistent with discontinuous or staggered junctions^{68,70} and the less prominent and even reverse refractive index gradients in this region.⁷ Band 1 is considered a marker for outer retinal integrity, under the assumption that it includes only the ELM.^{71,72} One explanation for the discrepancy between the anatomical ELM and band 1 thickness is the presence of additional reflectivity sources. Of note, mitochondria localize to Müller cells internal to the adherens junctions.^{73,74} If mitochondria can contribute reflectivity to band 2 without being long and bundled,^{47,48} then they could also contribute to band 1.

This study has limitations that are addressable in future research. The RefMoB was tested only in normal retinas with continuous bands. The OS length is diurnally variable, which we did not test.⁷⁵ The RefMoB is specifically designed for one instrument. Our volume scans did not extend to eccentricities in which band 3 is proposed to split. Fitting of two-dimensional and three-dimensional Gaussians would include more data and require less postprocess filtering. Any function that better describes the reflective response could replace the Gaussians, such as a Lorentzian function.⁴⁹ Better performance by manual evaluators could result from specific training.⁷⁶ Nevertheless, RefMoB is currently ideally suited for investigating the identity and regulation of subcellular reflectivity sources in eyes with minimally perturbed pathology. Accurate measures of four hyperreflective bands also provide accurate measures of the interleaved hyporeflexive bands, each of which has an associated anatomical correlate, protein expression, and retinal disorders of interest. Finally, 22% of older adults with healthy-appearing maculas have markedly slowed dark adaptation, consistent with an age-related impairment of transport across the RPE-BrM complex and a possible functional biomarker of early AMD^{42,77}; it is of interest to determine band 3 and 4 thicknesses in these eyes. By testing hypotheses with RefMoB, we can further improve the interpretation of SDOCT in the diagnosis and management of chorioretinal disease.

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