Extrafoveal Cone Packing in Eyes With a History of Retinopathy of Prematurity

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PURPOSE. To study the density and packing geometry of the extrafoveal cone photoreceptors in eyes with a history of retinopathy of prematurity (ROP). We used a multimodal combination of adaptive optics (AO) scanning light ophthalmoscopy (SLO) and optical coherence tomography (OCT).

METHODS. Cones were identified in subjects (aged 14-26 years) with a history of ROP that was either severe and treated by laser ablation of avascular peripheral retina (TROP; n = 5) or mild and spontaneously resolved, untreated (UROP; n = 5), and in term-born controls (CT; n = 8). The AO-SLO images were obtained at temporal eccentricities 4.5°, 9°, 13.5°, and 18° using both confocal and offset apertures with simultaneous, colocal OCT images. Effects of group, eccentricity, and aperture were evaluated and the modalities compared.

RESULTS. In the SLO images, cone density was lower and the packing pattern less regular in TROP, relative to CT and UROP retinae. Although SLO image quality appeared lower in TROP, root mean square (RMS) wavefront error did not differ among the groups. In TROP eyes, cone discrimination was easier in offset aperture images. There was no evidence of cone loss in the TROP OCT images.

CONCLUSIONS. Low cone density in TROP confocal SLO images may have resulted from lower image quality. Since AO correction in these eyes was equivalent to that of the control group, and OCT imaging showed no significant cone loss, the optical properties of the inner retina or properties of the cones themselves are likely altered in a way that affects photoreceptor imaging.

Keywords: retinopathy of prematurity, cones, physiological optics, image analysis, photoreceptor morphology

Long-term sequelae of retinopathy of prematurity (ROP) on cone-mediated visual function include low visual acuity, visual field loss, reduced contrast sensitivity, and altered color vision.1-13 The extent to which underlying retinal abnormality is associated with these vision deficits remains incompletely specified. Electroretinographic (ERG) studies, both full-field and multifocal, have demonstrated deficits in cone and cone-mediated retinal function in subjects with a history of ROP.14,15 Studies of retinal structure in ROP subjects have, on the other hand, shown little in the way of abnormalities of the photoreceptor laminae, although altered foveal architecture, such as shallow pit with no central avascular zone and thicker postreceptor retinal laminae, has been documented.1,2,16,17

We used a multimodal adaptive optics retinal imager (MAORI; Physical Sciences, Inc., Andover, MA, USA) to capture simultaneous scanning light ophthalmoscopical (SLO) and spectral-domain optical coherence tomographic (OCT) retinal images.16,18 The SLO was used to visualize individual cone photoreceptors en face and the OCT was used to obtain cross-sectional images of photoreceptor and postreceptor laminae.16,18 Confocal SLO poorly visualizes photoreceptors with abnormal outer segment morphology or alignment, whereas offset aperture SLO visualizes the cone mosaic, regardless, by collecting the light scattered by the cone inner segments.19,20 Because anatomic studies in a rat model of ROP indicate that the photoreceptors may be structurally disorganized,21 we used both confocal and offset aperture methods12-24 as well as OCT (an approach based upon low-coherence interferometry) to visualize the cones and measure their density. In the SLO images, we also ascertained cone packing geometry.

METHODS

Subjects

Subjects (aged 14-26 years) with a history of ROP were studied. All had serial fundus examinations in the newborn intensive care nursery similar to those used in the multicenter ROP
treatment trials. Based upon these examinations, every subject was grouped according to the maximum acute-phase ROP. For severe ROP (zone II, stage 3), the avascular peripheral retina was treated by laser ablation (TROP; n = 5). For mild ROP (zone II, stage 1), the untreated clinical disease resolved spontaneously (UROP; n = 5). No subject had zone I disease, a history of retinal detachment, or retinal surgery other than laser treatment. Healthy, term-born subjects were also studied as controls (CT; n = 8). Written, informed consent was obtained from all adult subjects (aged 18 years or older) and from the parents of minor subjects, with assent from the minors. The treatment of subjects conformed to the Declaration of Helsinki and was approved by the Boston Children’s Hospital Committee on Clinical Investigation.

In preparation for imaging, an ophthalmic examination was conducted that included visual acuity assessment using an Early Treatment Diabetic Retinopathy Study (ETDRS) chart, noncycloplegic autorefraction (WR-5100K; Grand Seiko, Hiroshima, Japan), ocular biometry (IOLMaster 500; Carl Zeiss Meditec AG, Jena, Germany; Sonomed E-Z Scan AB5500; Lake Success, NY, USA), and wide-field, volumetric OCT of the retina that are parfocal and colocal. For the subjects with more than 4 diopters (D) of myopia, a –5.00 D spherical lens was placed in the optical path at a pupil conjugate to remove low-order defocus and preserve the DM’s stroke for higher-order ocular aberrations. Scanning light ophthalmoscopy and OCT imaging were performed, simultaneously, in temporal retina at four eccentricities (4.5°, 9°, 13.5°, and 18°) along the transverse meridian. To image each eccentricity, the subject was instructed to fixate a target (a plus symbol) offset from the imaging raster. Because an approach to cone counting has not been established in eyes with diseased photoreceptors, SLO-confocal, SLO-offset pinhole, and OCT methods were used and compared. Images were first acquired using a 100-μm (~2 Airy disk)-diameter confocal pinhole and then using a 500-μm pinhole offset by ~6 Airy disk diameters, the direction of the offset was optimized by the operator to best visualize the cones. Two to four sets of SLO and OCT videos, each consisting of 64 frames at 1-megapixel (1024 × 1000) resolution, were recorded using both pinholes at each eccentricity. The SLOs captured 1° × 1° of retina, and OCTs were sagittal 1° B-scans consisting of 1024 A-scans.

SLO Image Analysis

From each SLO video, 10 to 40 frames were aligned, using a nonrigid registration algorithm, and averaged to obtain a single image for analysis in a custom program (MATLAB; The MathWorks, Natick, MA, USA). An experienced examiner selected, by eye, the 0.75° × 0.75° area that showed the highest-quality cone mosaic. The cropping was advantageous because imperfect fixation by the subjects led to uneven amounts of averaging over the field. Cone centroids were initially detected by the software (imextendedmax.m, MATLAB), and then the examiner added or removed centroids as needed. Cone density was initially scaled in cells·deg⁻². To scale density in cells·mm⁻², the “angular subtense” of 1° of retina was calculated for each individual subject by

\[ \text{Angular Subtense} = \tan(1\,^\circ) \times (\text{Axial Length} - NF'), \]

where \( NF' \) is the position of the secondary nodal point of the eye. To calculate \( NF' \) in each subject, Bennett’s step-along formulae were used. Each subject’s measured values of axial length, anterior corneal curvature, anterior chamber depth, and lens thickness were plugged into these formulae; other values were obtained from Gullstrand’s Schematic Eye No. 2.

Dividing initial cone density (cells·deg⁻²) by the square of angular subtense (mm²·deg⁻²) specified density in cells·mm⁻². At each eccentricity, every subject’s cone diameter was estimated as the mean of the major and minor axes of ellipses manually fit to 20 representative cones in each image. Cone packing geometry was assessed by applying Voronoi tessellation (voronoin.m, MATLAB) with the cone centroids as the generators. Voronoi cells that did not close, or appeared to close outside the image, were excluded from analysis.

OCT Image Analysis

Up to 45 B-scans from each OCT were aligned by cross-correlation and averaged. The final images were manually marked in ImageJ to delineate the boundary of the vitreous and nerve fiber layer (NFL), the boundary between the outer plexiform layer (OPL) and outer nuclear layer (ONL), and the boundary between the retinal pigment epithelium (RPE) and choroid. Thickness of the postreceptor (NFL-OPL) and photoreceptor (ONL-RPE) laminae in each OCT was taken as the mean across all Ascans.

In the OCT images, the cone photoreceptors were identified and counted by a second examiner. To produce a provisional measure of correspondence between the imaging modalities (SLO versus OCT), the square root of SLO cone density (cells·deg⁻¹) was calculated and plotted against the cones detected in the OCT (cells·deg⁻¹).

Wavefront Analyses

The DM is controlled by a Shack-Hartmann wavefront sensor (WS) composed of a lenslet array and a charge-coupled device camera (Uniq Vision, Inc., Santa Clara, CA, USA). A lens relay placed in front of the sensor blocks reflections from out-of-plane objects (e.g., the cornea), providing for high-quality correction to reflections from the retina. In order to evaluate the relative quality of the corrections in the three groups, videos of the WS spots were evaluated to determine their displacement from the plane-wave reference pattern, and a root mean square (RMS) error term was calculated for every scan in every subject; these were then averaged to produce a mean RMS for each subject.

Statistical Analyses

Respective 1-factor ANOVAs were used to evaluate differences among the three groups (TROP, UROP, CT) in spherical equivalent refraction, axial length, anterior chamber depth, lens thickness, angular subtense, and WS RMS error. The primary independent variables were group and eccentricity; but SLO-confocal, SLO-offset pinhole, and OCT methods were also compared. Full-factorial repeated-measures (subjects) mixed linear models were applied to the cone density, packing geometry, and inner segment diameter data, measured in SLO images, to evaluate the effects of group, eccentricity (4.5°, 9°, etc.), and eccentricity (4.5°, 9°, etc.).
13.5°, 18°), and aperture (confocal, offset). These were followed by Bonferroni-adjusted pairwise multiple comparison tests. Cone density, measured in the OCT, was evaluated using an additional mixed linear model with the same factors. A final mixed linear model, with an additional factor, layer (postreceptor, photoreceptor), was used to evaluate retinal thickness. The aforementioned analyses were all conducted in IBM SPSS Statistics 22 (SPSS; IBM, Chicago, IL, USA). Scanning light ophthalmoscopy and OCT cone counts were compared, using orthogonal regression, in Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). For all tests, the level of significance, z, was set at 0.05.

**RESULTS**

The Table lists visual acuity, spherical equivalent, ocular dimensions, and calculated angular subtense for each subject. Values in TROP differed significantly from those in UROP and CT in the following ways: Visual acuity was poorer ($P = 2.45 \times 10^{-4}$), spherical equivalent was more myopic ($P = 0.0158$), anterior corneal radius of curvature was steeper ($P = 5.18 \times 10^{-4}$), the depth of the anterior chamber was shallower ($P = 0.00343$), and lens thickness was greater ($P = 0.0137$). Axial length and angular subtense did not differ significantly among the groups. Post hoc testing indicated that UROP and CT did not differ in any of these

**TABLE.** Subject Characteristics

| Subject | Visual Acuity, logMAR | Spherical Equivalent, D | Axial Length, mm | Anterior Corneal Radius, mm | Anterior Chamber Depth, mm | Lens Thickness, mm | Angular Subtense, mm-deg$^{-1}$ |
|---------|----------------------|------------------------|-----------------|----------------------------|---------------------------|-------------------|-------------------------------|
| CT1     | +0.02                | +0.69                  | 22.4            | 7.49                       | 3.39                      | 3.77              | 0.261                         |
| CT2     | -0.08                | +0.38                  | 22.9            | 7.59                       | 3.43                      | 3.57              | 0.269                         |
| CT3     | -0.16                | -0.07                  | 23.3            | 7.50                       | 3.57                      | 4.02              | 0.275                         |
| CT4     | -0.10                | -0.38                  | 23.8            | 7.93                       | 3.73                      | 3.70              | 0.279                         |
| CT5     | -0.12                | -1.38                  | 24.1            | 8.04                       | 3.55                      | 3.77              | 0.284                         |
| CT6     | -0.14                | -1.19                  | 25.9            | 7.87                       | 3.53                      | 3.52              | 0.284                         |
| CT7     | -0.12                | -2.19                  | 23.8            | 7.59                       | 3.85                      | 3.55              | 0.283                         |
| CT8     | -0.18                | -4.50                  | 25.4            | 7.52                       | 4.31                      | 3.72              | 0.309                         |
| UROP1   | -0.20                | +0.81                  | 22.5            | 7.50                       | 4.01                      | 3.55              | 0.261                         |
| UROP2   | -0.08                | -0.25                  | 23.4            | 7.60                       | 3.55                      | 3.57              | 0.278                         |
| UROP3   | +0.04                | +0.13                  | 22.9            | 7.55                       | 3.76                      | 4.00              | 0.267                         |
| UROP4   | -0.06                | -1.51                  | 24.1            | 7.64                       | 4.03                      | 3.80              | 0.286                         |
| TROP1   | +0.16                | -1.81                  | 22.3            | 7.12                       | 3.43                      | 3.55              | 0.276                         |
| TROP2   | +0.04                | -2.15                  | 21.2            | 7.05                       | 3.28                      | 4.15              | 0.249                         |
| TROP3   | +0.16                | -3.38                  | 21.5            | 6.96                       | 2.71                      | 4.17              | 0.254                         |
| TROP4   | +0.04                | -4.82                  | 24.2            | 7.26                       | 3.59                      | 3.79              | 0.294                         |
| TROP5   | +0.10                | -5.13                  | 23.4            | 7.22                       | 2.80                      | 4.80              | 0.281                         |

logMAR, logarithm of the minimum angle of resolution.

**FIGURE 1.** Sample confocal aperture SLO images (0.25° × 0.25°) obtained at four eccentricities (top) from the subjects indicated (left).
parameters. The clinical OCT scans of TROP subjects showed the fovea centralis to be conspicuously shallow and the postreceptor laminae thickened; the photoreceptor laminae appeared normal. There was no significant difference in the WS RMS error among CT, UROP, and TROP.

Scanning Light Ophthalmoscopy

Figures 1 and 2 show representative registered and averaged images, obtained at the four eccentricities, using the confocal and offset apertures, respectively. At 18° and 13.5°, the cones appeared more readily discriminable when using the offset aperture versus the confocal aperture; there appeared to be more bright spots, with a range of sizes, in the confocal compared to offset aperture images. At 9° and 4.5°, the difference was less apparent.

Cone Density. Figure 3 shows the density of the cone photoreceptors, identified in individual subjects, using both the confocal aperture (left) and the offset aperture (right) at all eccentricities. There were significant main effects of eccentricity ($P = 4.90 \times 10^{-13}$), aperture ($P = 3.30 \times 10^{-4}$), and group ($P = 4.55 \times 10^{-10}$). Density decreased with increasing eccentricity.

![Figure 2](image2.png)

**Figure 2.** Sample offset aperture SLO images (0.25° × 0.25°) obtained at the same eccentricities (top) from the same subjects (left) as in Figure 1. Compared to the confocal images (Fig. 1), identification of the cones at 13.5° and 18° appeared less ambiguous when the offset aperture was used.

![Figure 3](image3.png)

**Figure 3.** Cone density measured from SLO images using the confocal aperture (left) and the offset aperture (right). Each symbol represents data from an individual subject. The line segments connect the group means at each eccentricity.
Density was also lower in the offset aperture SLOs. The difference between confocal and offset aperture density values is in the direction expected if rods contributed to the estimates of density in the former. According to post hoc analyses, the group effect was driven by the TROP images, which contained significantly fewer cones than the CT or UROP images (which, in turn, did not differ from one another). There were no significant interaction effects.

**Cone Diameter.** Fitted cone diameter did not vary significantly with either group or eccentricity. However, it was significantly higher \((P = 0.0438)\) when using the offset aperture \((\text{range: } 7.71–11.2 \mu m; \text{median: } 8.88 \mu m)\) compared to the confocal aperture \((\text{range: } 7.05–12.0 \mu m; \text{median: } 8.57 \mu m)\).
The offset aperture yielded images with well-circumscribed, “bubble wrap” appearance of cones, the diameters of which were quite similar to those obtained in anatomical studies. 37

Cone Packing Geometry. In Figure 4A, the Voronoi tessellation is superimposed on the 18° offset aperture image of subject CT4. A normal distribution summarizes the side counts in Figure 4B. For this and all other Voronoi tessellations, the mean number of sides was close to six. The standard deviation in the number of sides (Figs. 4C, 4D), however, varied with group ($P = 9.84 \times 10^{-7}$), eccentricity ($P = 2.12 \times 10^{-6}$), and aperture ($P = 0.0463$). Post hoc, between-group comparisons found that the standard deviation was significantly higher in TROP than in either UROP or CT images. This is consistent with irregular cone packing in TROP eyes. Moreover, a significant eccentricity × aperture interaction ($P = 0.00118$) indicated more variability in the cone packing patterns displayed in eccentric confocal scans.

Optical Coherence Tomography

Counts of cones visible in the OCT images (Fig. 5A) were obtained in both the offset and confocal configurations.

Retinal Thickness. The photoreceptor and postreceptor laminae are demarked on a sample image in Figure 5A. Figure 5B shows the mean photoreceptor and postreceptor retinal thickness for each group and eccentricity. Unsurprisingly, total retinal thickness decreased significantly with increasing eccentricity ($P = 1.93 \times 10^{-33}$). Total retinal thickness differed significantly among groups ($P = 0.00665$); both UROP and TROP retinae were significantly thicker than CT retinae in post

![Figure 5](https://example.com/figure5.png)

**FIGURE 5.** Optical coherence tomography image analyses. (A) Sample OCT from 18° eccentric in subject CT4. Lines mark the vitreoretinal boundary (top), OPL–ONL boundary (middle), and RPE–choroid boundary (bottom). At the ellipsoid zone, bright cone inner segments are identified (arrows). (B) Stacked mean thickness of the photoreceptor (solid) and postreceptor (crosshatched) retinal layers for each group at the four eccentricities. (C) Cones counted in the OCT images. The symbols indicate the individual subjects as in Figures 3 and 4. The line segments connect the group means at each eccentricity.

![Figure 6](https://example.com/figure6.png)

**FIGURE 6.** Comparison of OCT and SLO cone counts obtained using the confocal aperture (left) and offset aperture (right). The symbols indicate the same subjects as in Figures 3, 4, and 5. The lines are orthogonal regressions through the data for each group. The scatter of points around the regression lines for TROP and UROP subjects is greater with the confocal than the offset aperture.
Extrafoveal Cone Packing in Eyes With ROP

The cones in extrafoveal TROP retina appeared to be reduced in density and irregularly packed, relative to those in CT and UROP. Although the present study does not include data from former preterms without a history of ROP that the UROP subjects did not differ from the CT subjects makes it very unlikely that their inclusion would have changed our conclusions.

Cone density in CT subjects, whether obtained using the confocal or offset aperture, was in reasonable agreement with anatomic data. For example, at 4.5° temporal eccentricity, Curcio et al.37 show ~15,000 cones-mm⁻²; in our sample, mean CT cone density at this location was 13,641 (range, 10,679–17,566) cones/mm² using the confocal aperture and 11,407 (range, 9,221–13,981) cones/mm² using the offset aperture. We suspect that we were failing to identify many cones in the confocal images, compensated by the erroneous cones in extrafoveal TROP retina that was responsible for the increased total thickness in TROP (P = 4.64 × 10⁻⁸), a result that keeps company with ERG38 and psychophysical39 data suggesting that beneficial remodeling of the inner retina occurs in eyes with a history of ROP.

**Cone Counts.** Figure 5C shows the number of cones (cells-deg⁻¹) at each eccentricity, identified using the OCT, in individual subjects. The only significant effect (main or interaction) was the main effect of eccentricity (P = 2.38 × 10⁻¹⁴). As eccentricity increased, cone density decreased. No group differences were detected.

Cone counts obtained by OCT are compared, by orthogonal regression, to the square roots of those obtained by SLO, in Figure 6. In CT subjects, cone counts by OCT and SLO were about the same, whether the confocal or offset aperture was used, and the regressions were significant (confocal P = 4.76 × 10⁻³; offset P = 1.18 × 10⁻⁴). Only when using the offset aperture were the regressions significant for UROP (P = 7.89 × 10⁻⁴) and TROP (P = 0.0284). With the offset aperture, the slopes of the regressions were nearly the same but the elevations (intercepts) were significantly different (P = 0.00105). With the confocal aperture, due to increased scatter around the regression lines, the UROP and TROP regressions were not significant.

**Discussion**

The cones in extrafoveal TROP retina appeared to be reduced in density and irregularly packed, relative to those in CT and UROP. Although the present study does not include data from former preterms without a history of ROP that the UROP subjects did not differ from the CT subjects makes it very unlikely that their inclusion would have changed our conclusions.

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The offset and confocal apertures offer complementary detection schemes that emphasize different local optical properties of imaged structures: The confocal aperture favors visualization of strongly backscattering structures and retinal layer interfaces, such as the cone inner-segment/outer-segment junctions and the outer segment tips. The offset aperture enables better visualization of more subtle forward- and multiply-scattering structures, such as the complicated structures of the cone inner segments.19,23,28,47,48 This has important implications for imaging diseased retina because confocal imaging methods may obscure the source of the optical changes exhibited by abnormal photoreceptors.20 That said, TROP cone counts were lower regardless of aperture (confocal, offset).

Most cones identified in the TROP SLOs were more irregularly arranged, as indicated by Voronoi tessellation of their centroids. Regularly arranged cones have predominantly hexagonal packing, indicated by mean number of sides close to six and low standard deviation.49 High standard deviation in the number of sides in the TROP tessellations indicates disruption of normal cone organization. However, the same image quality caveats mentioned above apply to this measure of local order. We are unaware of any standard “noise-equivalent” order analysis by which to gauge the significance of this finding.

Indeed, the OCT showed that the thickness of the photoreceptor laminae in TROP subjects was normal. Further, the cone counts derived from the OCT did not differ among the groups or with respect to the aperture. Due to its primary sensitivity to directly back-scattered ballistic photons, OCT presumably does not depend so heavily on the other light-scattering properties of the cones as does SLO. Thus, the OCT result favors low image quality (rather than actual loss of cones) as the explanation for decreased cone density in TROP SLOs. However, caution in interpretation of the OCT cone counts is warranted: The random interconnections of cones along individual B-scan planes as they shift on the retina (due to imperfect fixation) make this indirect, linear measure of cone density (cones-mm⁻²) intrinsically less precise than en face SLO area cone counts (cones-mm⁻²). Taken together, the imaging data reinforce the notion that severe ROP is associated with persistent changes in the photoreceptors,1 likely affecting their optical properties. Further, the thickening of the postreceptor laminae in TROP suggests that inner retinal cells remodel compensatorily to loss of photoreceptor input.

More, and more robust, imaging modalities with cross-modal comparison can advance visualization and understanding of retinal structure. Such methods may be especially important for investigation of diseased eyes.

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