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ORIGINAL ARTICLE

Directional Optical Coherence Tomography Reveals Reliable Outer Nuclear Layer Measurements

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ABSTRACT

Purpose. Directional Optical Coherence Tomography (D-OCT) is a method used to optically segment and identify the outer nuclear layer (ONL) *in vivo*. The purpose of this study was to determine the repeatability and reproducibility of D-OCT ONL thickness measurements in healthy eyes.

Methods. Sixteen healthy eyes of sixteen subjects were imaged using the Cirrus SD-OCT. The OCT beam entry position was varied horizontally and vertically through the pupil, and cross-sectional images were obtained at baseline and 1-month follow-up by two observers. Detailed segmentation was performed to quantify the thickness of ONL without the inclusion of overlying Henle Fiber Layer. Inter-observer, intra-observer, and inter-visit variability was evaluated using Bland-Altman and coefficient of variation analysis for each category.

Results. All 16 eyes were successfully imaged, registered, and segmented. The maximum mean (SD) inter-operator difference was 2.6 (4.8) μm . The maximum mean (SD) intra-operator difference was 2.4 (5.3) μm . There was no statistically significant difference in ONL measurements detected between baseline and follow-up ($p > 0.05$). The mean (SD) differences measured across visits by one operator varied from -1.6 (3.1) to 1.1 (6.1) μm . The mean (SD) coefficient of variance (CV%) for all sectors with horizontal orientation was 9.1% (2.3%), 10.1% (2.5%), and 8.6% (2.3%) for inter-observer, intra-observer, and inter-visit, respectively. The mean (SD) coefficient of variance (CV%) for all sectors with vertical orientation was 8.3% (1.8%), 6.9% (1.4%), and 8.3% (2.1%) for inter-observer, intra-observer, and inter-visit, respectively. The majority of the variation of paired repeated measurements originated from between-subject variance. The within-subject variance accounted for less than 1% of the total variability.

Conclusions. ONL thickness measurements can be quantified with good repeatability and reproducibility using D-OCT. Identifying the magnitude of D-OCT variability among normal subjects will allow for improved development of future clinical studies that quantitatively track the progression of macular pathology. (Optom Vis Sci 2016;93:714–719)

Key Words: ocular coherence tomography, directional OCT, repeatability, reproducibility, Henle fiber layer, outer nuclear layer, retina

The retinal outer nuclear layer (ONL) contains photoreceptor cell bodies,¹ which because of their direct relationship to visual function is a critical biomarker of macular disease. The thickness of ONL has been measured histologically in studies

monitoring the natural history and therapeutic interventions of retinal diseases including macular degeneration,^{2,3} Stargardt disease,⁴ and retinitis pigmentosa.^{5,6}

Optical coherence tomography (OCT) uses the reflectivity of infrared light to provide *in vivo* cross-sectional visualization of the retina previously only attainable histologically.⁷ Despite advancements in OCT technology and signal processing, macular ONL cannot be reliably visualized because of its lack of contrast with Henle fiber layer (HFL) with standard on-axis scans.⁸ Even after adjustment to brightness and contrast of OCT scans, the identification of the boundaries has not been shown to be precise and reproducible among different graders.⁹ Despite this, other

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FIGURE 1.

OCT HD5 Line Raster scans were acquired in three locations along the horizontal and three locations along the vertical.

studies in the past have looked at the cross-sectional ONL thickness in Stargardt disease,¹⁰ achromatopsia,¹¹ and central serous chorioretinopathy¹² using traditional OCT.

Directional OCT (D-OCT) is a novel technique which obtains images of the same part of the retina from multiple pupil entry positions of the OCT beam to take advantage of the directionally reflective components of the retina, specifically HFL and the hyper-reflective outer retinal photoreceptor bands. By capitalizing on the oblique orientation of HFL, displacing the pupil entry position laterally causes the contralateral HFL to become hyper-reflective and the ipsilateral HFL to become hypo-reflective. Because of the radial symmetry of HFL, the same principle can be applied to scans acquired through any lateral pupil entry position.¹³ D-OCT causes a change in HFL reflectivity which adds optical contrast and has been shown to provide repeatable and reproducible measurements of the true ONL thickness when obtained by a single experienced operator.¹⁴

For D-OCT to be integrated into future clinical studies, it must be easily obtainable by experienced and inexperienced operators alike, and ONL measurements must be reliable. This study aims to determine the inter-operator, intra-operator, and inter-visit repeatability and reproducibility of D-OCT ONL thickness measurements in healthy eyes.

METHODS

Subjects were recruited from the UC Berkeley School of Optometry Eye Center and provided informed consent before participation in this study. Subjects ranged from 40 to 80 years of age and were categorized into 40s, 50s, 60s, and 70s age groups, with a total of 4 subjects per group. One eye per subject was studied, which was required to have a BCVA of at least 20/25 with refractive error less than -7.00D . Sufficiently clear media for imaging, good fixation, and absence of any macular pathology as determined by review of an undilated OCT scan by a retinal specialist were also required for inclusion.

Eyes were dilated with 1% tropicamide and 2.5% phenylephrine. A series of OCT scans were then performed by two operators using Cirrus HD-OCT 4000. Operator 1 had extensive previous experience acquiring D-OCT scans. Operator 2 was given a protocol and a brief training session.

OCT scans included HD5 Line Raster (0 mm spacing) acquired through three entry positions for both vertical and horizontal (Fig. 1) and with and without utilization of the tracking software within the instrument that purports to compensate for small eye movements or blinks. The operator order, scan order, and tracking versus no tracking order were decided by a pre-determined block randomization. To standardize the displacement, each direction received “two clicks” at the edge of the crosshair (Fig. 2), a distance previously demonstrated to produce and unambiguous ONL/HFL boundary.¹³ In instances where two clicks would degrade the image quality, the entry point was moved back such that the image was still tilted but would produce the best OCT quality possible. The HD5 Line Raster “flat” scan was performed in the 180° and 90° meridians by ensuring the image appeared symmetrical about the fovea in each meridian. After each set of scans, the subject was instructed to take a break outside of the machine, which would reset the OCT. After a short break, the subject would be realigned to begin the next set. Operator 2 was not present while operator 1 was performing scans, and the results were not discussed during the transition between operators. At the follow-up visit, the same eye was dilated and an axial length measurement was taken, and only operator 2 took the same sets of D-OCT scans as at the initial visit.

Bitmap images of the HD5 Line Raster (0 mm spacing) were registered through custom MATLAB software (R2012a; The MathWorks, Natick, MA), which aligned the tilted images with its respective “flat” image based upon the contour of the internal limiting membrane (ILM) of the flat horizontal and flat vertical images.¹⁵ These were then separated into groups based upon horizontal or vertical, tracking or no tracking, and operator 1 or operator 2. Each group contained three images, which were stacked using ImageJ (U.S. National Institute of Health, Bethesda, MD) and a total of 200 points were carefully applied to the image. Each of the following layers of the retina contained

FIGURE 2.

Pupil entry position was standardized such that each direction would receive “two clicks” at the location indicated at the tip of the arrow from the flat entry position.

40 points: the top of the ILM, the top of HFL, the bottom of HFL, the bottom of the external limiting membrane (ELM), and the retinal pigment epithelium (RPE). The points placed at the top of the ILM, the ELM, and the RPE were based on the true “flat” image. The top and bottom of the HFL were placed based upon the appropriate tilt that offered contrast between the HFL and ONL (Fig. 3). A MATLAB curve fitting function was then used to standardize analysis between subjects to consistent eccentricities along each segmented layer.¹⁴

The thickness of ONL was assessed at 0.1 mm intervals inside a 3 mm radius. The mean and standard deviation (SD) of ONL thicknesses were obtained for center (0.5 mm radius), midperiphery (0.5–1.5 mm radius), and periphery (1.5–3.0 mm radius) along both horizontal and vertical meridians.

RESULTS

Sixteen healthy eyes of sixteen subjects were successfully imaged, registered, and segmented. The primary area of concern was ONL and HFL, thus thickness measurements were obtained using only points at the top and bottom of HFL, and at the external limiting membrane (Fig. 3). Intra-operator, inter-operator, and inter-visit variability were analyzed following Bland-Altman graphical approach. Then 95% limits of agreement (LOA) were calculated and defined as mean difference $\pm$ 1.96 SD, which indicated that between-measurement differences should lie within the limits 95% of the time. The smaller the range between the limits, the better the agreement (Fig. 4). Inter-operator variability was analyzed using data from visit 1 and inter-visit variability was analyzed using data from observer 2.

The maximum mean (SD) inter-operator difference was 2.6 (4.8) μm . The maximum mean (SD) intra-operator difference was 2.4 (5.3) μm . There was no statistically significant difference in ONL measurements detected between baseline and follow-up ($p > 0.05$) (Table 1). The mean (SD) differences measured across visits by one operator varied from -1.6 (3.1) to 1.1 (6.1) μm (Table 2).

For all subjects, fixation was excellent, and all scan sets could be aligned using the HD5-Line 0 mm setting. There was no difference found between scans acquired using the tracking setting and scans acquired without tracking.

Side-by-side agreement was compared by calculating coefficient of variation (CV%). The smaller the CV%, the smaller the variance. The mean CV% (SD) for all sectors with horizontal orientation was calculated to be 9.1% (2.3%), 10.1% (2.5%), and 8.6% (2.3%) for inter-operator, intra-operator, and inter-visit, respectively. The mean (SD) CV% for all sectors with vertical orientation was 8.3% (1.8%), 6.9% (1.4%), and 8.3% (2.1%) for inter-operator, intra-operator, and inter-visit, respectively.

In general, the older age group (60–73, $n = 8$) was found to have thicker ONL than the younger age group (40–59, $n = 8$) at baseline and follow-up. The difference was more apparent in the central zone than in mid-peripheral and peripheral area, ranging from 5.8 to 8.0 μm ; however, these differences were not statistically significant.

A post hoc power calculation was performed using descriptive statistics from the study. The correlation from all paired observations was calculated as 0.90 with a significance level set to 0.05. The overall averaged SDs for each zone were used and a total of 16 subjects achieved a power of 0.83 to detect a minimum difference of 4 μm from repeated measurements and a power of 0.95 to detect a minimum difference of 5 μm for central ONL.

FIGURE 3.

Segmentation of varying scans obtained from a right eye. A, Center entry position with 40 points on the ELM and a poorly visualized HFL. B, Nasal entry position showing hyperreflectivity of the temporal HFL and hyporeflectivity of the nasal HFL. 40 points placed at each of the following: top of HFL, bottom of HFL, and ELM. C, Temporal entry position showing hyperreflectivity of the nasal HFL and hyporeflectivity of the temporal HFL. 40 points placed at each of the following: top of HFL, bottom of HFL, and ELM.

FIGURE 4.

Difference versus mean Bland-Altman plots. Center red line represents the mean of the difference from observers for the central ONL. Outer red lines represent 95% LOA for central ONL. Dashed blue lines represent 95% LOA for nasal mid-peripheral ONL. Dashed gray lines represent 95% LOA for temporal mid-peripheral ONL. The plotted points only represent central ONL thickness. The narrow width of LOAs and small mean difference values imply a good agreement for inter-observer, intra-observer, and inter-visit variability.

The power to detect a minimum difference of 4 μm for mid-peripheral and peripheral ONL reached as high as 0.99.

DISCUSSION

Directional OCT provides a useful method to clearly distinguish ONL from HFL *in vivo*, which was previously only attainable histologically. Repeatable measurements of ONL thickness, without the inclusion of HFL, are important for future clinical studies to

quantify macular photoreceptor nuclei thickness. The results of this study establish that ONL thickness measurements, without inclusion of HFL, can be quantified with good repeatability and reproducibility using D-OCT, and that the technique can be correctly performed with minimal additional training.

Our study found that the mean inter- and intra-operator differences were less than 3 μm across each of the retinal locations studied. This translates into less than one pixel on the Cirrus instrument. Including standard deviations, this approaches two pixels in difference for each of these measures. These results were comparable to other papers assessing repeatability and reproducibility for macular thickness and nerve fiber layer thickness,¹⁶ as well as within other papers measuring ONL while including HFL.¹⁷ The overall degree of variability was far less than between different OCT devices.¹⁸

The ONL comprised mostly rod and cone photoreceptor nuclei, along with Müller cell axons,¹ which makes it a critical layer to measure, as loss of these cell bodies have a direct correlation to retinal function.² ONL and HFL should be considered as independent layers because HFL has been found to increase in thickness with age,¹⁹ despite a 30% loss of macular rods throughout adulthood.²⁰ This may be why previous studies had found no significant change in the thickness of ONL between healthy subjects and subjects with early age-related macular degeneration,²¹ despite documented evidence of photoreceptor loss in this disease.^{2,3} Independent assessments of each of these layers may reveal a more complete understanding of how these diseases lead to vision loss.

Studies examining retinal changes due to ABCA4-associated retinal degenerations, such as Stargardt disease/fundus flavimaculatus, found some study cohorts to have substantial thinning of ONL, whereas others displayed no significant change. In this study, the measurements of ONL included HFL, thus they are likely under-representing the amount of ONL loss. The authors address this and agree that future studies should include D-OCT to separate the layers.¹⁰ A recent study evaluated the effectiveness of reduced fluence photodynamic therapy for the treatment of central serous chorioretinopathy and found that ONL thickness was positively correlated with visual prognosis.²²

In our cohort, the ONL thickness was found to be greater in the older age groups than in the younger groups. However, this difference was found to be not statistically significant and is likely due only to inter-subject variation in the population. While demonstrating that D-OCT could be performed on an older cohort of subjects in an age group that is more susceptible to macular diseases such as age-related macular degeneration was novel, the small sample size is a clear limitation of our study. The repeatability and reproducibility metrics established by this study justifies a larger D-OCT trial powered to investigate differences in ONL with age further.

A limitation of the D-OCT technique presented in this paper was the requirement for pupil dilation. The minimum deviation of the OCT beam required to be sensitive to directionally reflective changes was not sought or assessed in this study. Rather, unambiguous contrast between HFL and ONL was sought thereby taking advantage of a dilated pupil. Although dilation is safe when subjects are adequately screened, and there is no evidence to suggest that it would alter retinal thickness measurements, the requirement for dilation may be a practical limitation to the current technique.

TABLE 1.

Thicknesses of ONL (μm) at each location measured at baseline and 1 month in the horizontal and vertical meridians (SD in parentheses)

Horizontal meridian					
Visit	Nasal Periphery	Nasal Midperiphery	Center	Temporal Midperiphery	Temporal Periphery
Baseline	48.3 (6.2)	45.3 (6.4)	82.5 (11.1)	43.2 (7.2)	49.0 (7.5)
1 mo	50.0 (6.7)	45.6 (7.0)	80.8 (12.2)	44.1 (6.8)	50.3 (6.3)
Vertical meridian					
Visit	Inferior Periphery	Inferior Midperiphery	Center	Superior Midperiphery	Superior Periphery
Baseline	53.7 (6.7)	42.7 (7.2)	80.2 (11.7)	42.8 (7.6)	53.3 (10.0)
1 mo	55.1 (8.6)	43.5 (6.1)	78.7 (11.0)	44.1 (7.3)	56.6 (12.0)

N at baseline visit = 64; N at 1 month visit = 32.

TABLE 2.

Mean differences (μm) for inter-observer, intra-observer, and inter-visit at each sector (SD in parentheses)

Horizontal meridian			
Sector	Inter-observer	Intra-observer	Inter-visit
Center	0.9 (5.5)	1.7 (6.3)	−0.2 (8.5)
N-midperiphery	0.1 (5.3)	−0.6 (3.4)	0.7 (3.5)
N-periphery	−1.2 (5.6)	−2.4 (5.3)	0.8 (5.7)
T-midperiphery	0.1 (3.4)	0.2 (5.6)	1.1 (2.6)
T-periphery	−0.7 (3.9)	1.2 (5.9)	0.9 (3.8)
Vertical meridian			
Sector	Inter-observer	Intra-observer	Inter-visit
Center	2.6 (4.8)	0.2 (5.5)	−0.7 (6.6)
I-midperiphery	0.2 (4.3)	−0.6 (3.1)	−0.1 (3.7)
I-periphery	−2.0 (3.8)	−0.8 (2.7)	−1.6 (3.1)
S-midperiphery	0.5 (4.4)	−0.5 (2.7)	1.0 (3.2)
S-periphery	−0.3 (4.5)	0.9 (4.7)	1.1 (6.1)

This study utilized the Zeiss Cirrus instrument; however, this technique could be performed using other OCT systems as long as a “live” image of the B-scan is visible to the operator. Moreno et al. performed D-OCT measurements utilizing the Heidelberg Spectralis system by laterally moving the OCT arm while observing the HFL and acquiring images when the HFL/ONL interface was unambiguously visible.²³ An advantage of the Cirrus system in our study was that a novice operator could use the integrated pupil camera to facilitate D-OCT acquisition rather than having to interpret retinal anatomy while acquiring scans.

The results of this study indicate with high certainty that within a given individual, a greater than 5 μm difference in central ONL thickness or 4 μm difference in mid-peripheral or peripheral macular ONL thickness can be attributed to disease rather than variation in the acquisition or analysis technique. This establishes ONL, without inclusion of HFL, as an important biomarker for future studies looking to monitor disease progression or treatment efficacy.

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