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# Reproducibility and Repeatability of Ganglion Cell-Inner Plexiform Layer Thickness Measurements in Healthy Subjects

Paolo Carpineto Agbeanda Aharrh-Gnama Vincenzo Ciciarelli Alessandra Mastropasqua Luca Di Antonio Lisa Toto

Ophthalmology Clinic, University 'G. d'Annunzio' Chieti-Pescara, Chieti, Italy

## **Key Words**

Glaucoma diagnostics  $\cdot$  Macula  $\cdot$  Optical coherence tomography

#### **Abstract**

Purpose: To assess the reproducibility and repeatability of macular ganglion cell-inner plexiform layer (GC-IPL) thickness measurements in healthy subjects. **Procedures:** In this observational study, 60 healthy eyes were subjected to macular GC-IPL thickness measurements by means of Cirrus™ high-definition optical coherence tomography (Cirrus version 6.0; Carl Zeiss Meditec, Dublin, Calif., USA) by two examiners in two sessions. Average, minimum and 6 sectoral GC-IPL thicknesses were measured. Inter- and intraobserver reproducibility was tested and analyzed by means of the concordance correlation coefficient (CCC). The repeatability of measurements was assessed by the coefficient of repeatability (CR). Results: Mean age (±SD) was 29.63 (±5.1) years. The CRs for average GC-IPL thickness were 2.1 and 2.2 µm for the first and the second operator, respectively. Inter- and intraobserver CCCs ranged from 0.91 (95% CI: 0.89-0.93) to 0.98 (95% CI: 0.96-0.99) and from 0.92 (95% CI: 0.88-0.94) to 0.98 (95% CI: 0.97–0.99), respectively. **Conclusions:** GC-IPL thickness measurements in young healthy subjects showed excellent reproducibility and repeatability, especially for average and sectoral GC-IPL thickness measurements.

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#### Introduction

High-definition optical coherence tomography (HD-OCT) is a widely used imaging method for assessing the peripapillary retinal nerve fiber layer (RNFL) thickness, optic nerve head and macula. HD-OCT can be used to obtain images of the retina and its various anatomical layers with a high resolution and a good reproducibility [1, 2].

Recent advances in segmentation algorithms have increased the ability of separating and demarcating individual retinal layers [2]. The retinal ganglion cell layer (GCL) was reported to be the early site of glaucomatous damage, as shown in experimental models [3, 4]. Recent studies also showed that the segregated ganglion cell complex (GCC), which in the macular regions is the sum of RNFL, GCL and inner plexiform layer (IPL), has a similar capacity of discriminating glaucoma compared with RNFL thickness [5]. In addition, in nonarteritic ischemic optic neuropathy, hemispheric GCC loss correlated with altitudinal visual field loss [6], and in patients with minimal diabetic retinopathy, the GCL in the pericentral area of the macula was thinner than in normal control subjects [7, 8].

The latest HD-OCT ganglion cell analysis (GCA) algorithm (Cirrus version 6.0; Carl Zeiss Meditec, Dublin, Calif., USA) can successfully demarcate the macular ganglion cell-IPL (GC-IPL) while excluding the RNFL.

Reproducibility and repeatability are two of the main principles upon which scientific method are based and are an indicator of the applicability of any instrument as a diagnostic tool in clinical practice. They refer to the degree of agreement between independent measurements obtained with the same method/instrument on identical test material under different conditions (i.e., different operators) and under the same condition (i.e., same operator in consecutive measurements), respectively.

Recent studies [2,9,10] reported excellent intersession and intervisit reproducibility of macular GC-IPL measurement performed by a single operator both for healthy and glaucomatous eyes.

The aim of this study was to evaluate both the intra- and intersession reproducibility and the repeatability of macular GC-IPL thickness measurements performed by two operators using Cirrus<sup>TM</sup> HD-OCT in healthy subjects.

#### **Patients and Methods**

The study was performed at the Ophthalmology Clinic of the University of Chieti-Pescara, Italy. A total of 60 healthy volunteers aged between 19 and 49 years were enrolled in the study. The study adhered to the tenets of the Declaration of Helsinki and was approved by the local Ethics Committee. Informed consent was obtained prior to the session.

Each subject underwent a comprehensive ophthalmic examination, including a review of the medical history, best-corrected visual acuity (using an Early Treatment Diabetic Retinopathy Study chart), slit-lamp biomicroscopy, intraocular pressure measurement with Goldmann applanation tonometry, central corneal pachymetry, gonioscopy, dilated funduscopic examination using a 78-dpt lens and visual field testing. Humphrey 30-2 Swedish interactive thresholding algorithm (Carl Zeiss Meditec) visual field testing was used.

To be included, subjects had to have the following: (1) a bestcorrected visual acuity of 20/25 or better, (2) spherical refraction within  $\pm 3.0$  dpt, (3) cylinder correction within  $\pm 2.0$  dpt, (4) open angles on gonioscopy (Shaffer classification), (5) intraocular pressure of 21 mm Hg or less, (6) no conditions that increase the risk for secondary glaucoma (pigment dispersion syndrome or pseudoexfoliation) and (7) a healthy appearance of the macula lutea, the optic disc and the RNFL (no diffuse or focal rim thinning, cupping, optic disc hemorrhage, or RNFL defects), as evaluated by funduscopic examination. Subjects were declared healthy if visual field mean deviation, pattern standard deviation (SD) and the glaucoma hemifield test were all within normal limits for at least 2 consecutive reliable visual field tests obtained within 3 months from the OCT examination. Visual fields were considered reliable if fixation loss and false-negative and false-positive results were less than 30%. Subjects were excluded for the following reasons: (1) if they had any previous history of ocular disease, surgery, laser or medical treatments, or any systemic disease with ocular involvement, (2) if visual field mean deviation or pattern SD was below 5% cutoffs or (3) if the glaucoma hemifield test was outside normal limits in at least

2 reliable visual field tests. If the glaucoma hemifield test was borderline, mean and pattern SD were <10% but >5% or defects were inconsistent between visual fields, the subject was categorized as 'glaucoma suspect' and was removed from the data set.

Eyes with optical medium opacity, maculopathy, retinal disease, uveitis, or glaucomatous or nonglaucomatous optic neuropathy were excluded from the investigation.

Imaging with Cirrus HD-OCT

The Cirrus HD-OCT used in this study is a commercially available device with a scan speed of 27,000 axial scans per second and an axial resolution of 5 µm [11]. After pupil dilation using tropicamide 1% and phenylephrine hydrochloride 2.5%, Cirrus HD-OCT was used to acquire 1 macular scan using the macular cube  $512 \times 128$  scan protocol in each study eye. The GCA algorithm, incorporated in Cirrus HD-OCT software version 6.5, was used to process and measure the thickness of macular GC-IPL within a 14.13-mm<sup>2</sup> elliptical annulus area centered on the fovea. The GCA algorithm automatically segmented the GC-IPL based on the three-dimensional data generated from the macular cube 512 × 128 scan protocol. The outer boundary of the RNFL and the outer boundary of the IPL at the macular region were segmented by the algorithm; the segmented layer thus yielded a measurement of the GC-IPL thickness. The average, minimum and 6 sectoral GC-IPL thicknesses (superotemporal, superior, superonasal, inferonasal, inferior, and inferotemporal) were measured from the elliptical annulus centered on the fovea. The elliptical annulus has the following dimensions: vertical inner and outer radius of 0.5 and 2.0 mm, respectively, and horizontal inner and outer radius of 0.6 and 2.4 mm, respectively. This size and shape of elliptical annulus were chosen as they conform closely to the real macular anatomy, and the annulus corresponds to the area where the retinal GCL is thickest in normal eyes [2, 12].

The minimum GC-IPL measurement was determined by sampling 360 spokes of measurements extending from the center of the fovea to the edge of the elliptical annulus in 1-degree intervals and selecting the spoke with the lowest average.

A detailed description of the GCA scanning was reported elsewhere [2]. In brief, during image acquisition, the subject's pupil was first centered and focused in the iris viewport and the linescanning ophthalmoscope with "auto focus" mode was then used to optimize the view of the retina. The "center" and "enhance" modes were used to optimize the Z-offset and scan polarization, respectively, for the OCT scan in order to maximize the OCT signal. After each capture, motion artifacts were checked with the line-scanning ophthalmoscope image with an en face OCT overlay. Rescanning was performed if a motion artifact (indicated by blood vessel discontinuity) was detected. The HD-OCT scans were excluded if the retinal layer algorithm segmentation failed. All HD-OCT scans included in the study had a signal strength of at least 6, and we selected 1 eye from each participant randomly for final analysis.

Two different sessions of measurements were performed in 1 randomly selected eye of each subject at time 0 and after 30 days; the randomization was generated by using the Random Number Generator Pro 1.89, a free software available online.

In the first session 2 measurements of GC-IPL thickness were made using the Cirrus HD-OCT unit, each by two different examiners who had been trained in the use of the device. After 1 h, measurements were repeated in the same fashion but inverting the order of the examiners.

At the end of the first session, each eye had received 2 macular scans by each examiner. At the second session, the scans were repeated by the same two examiners using the same Cirrus HD-OCT unit. To minimize systematic bias, both examiners were masked with respect to subjects. After each scan acquisition, the examiners had no information on measurements at their disposal. GC-IPL thickness measurements were calculated only at the end of the second session.

An internal fixation light was used to center the scanning area on the fovea. Only scans with no interfering noise or artifacts were included in the study, and each individual scan was reviewed for aberrant placement of the inner and outer GC-IPL borders.

Scans with low quality and a failing GC-IPL thickness algorithm were excluded, and measurements were repeated until good quality was achieved. The quality score had to be higher than 6 out of 10. In addition, scans with blinks during the scanning process were excluded and repeated.

#### Statistical Analysis

The D'Agostino-Pearson test that computes a single p value for the combination of the coefficients of skewness and kurtosis was used to study the sample distribution. Intra- and intersession reproducibility was evaluated by means of the concordance correlation coefficient (CCC). The CCC evaluates the degree to which pairs of observations fall on the 45-degree line through the origin [13]. It contains a measurement of precision  $\rho$  (the Pearson correlation coefficient, which measures how far each observation deviates from the best-fit line) and accuracy  $C_b$  (a bias correction factor that measures how far the best-fit line deviates from the 45-degree line through the origin):  $\rho_c = \rho C_b$ .

The Bland-Altman plot was used to assess the repeatability of the method by comparing repeated measurements for each single examiner [14]. Since the same method is used for the repeated measurements, the mean difference should be zero. The coefficient of repeatability (CR) was calculated as 1.96 (approx. 2) times the SD of the differences between the measurements.

### Results

A total of 60 eyes of 60 subjects (21 males and 39 females) underwent GC-IPL thickness measurements with the previous described protocol. All the eyes that provided high-quality scans were prospectively included in the study. The sample age ranged from 19 to 49 years (mean 29.63 years, SD  $\pm$ 5.1 years). Additional demographic and clinical characteristics of the enrolled subjects are reported in table 1. GC-IPL thickness measurements are shown in table 2.

Average GC-IPL thickness data showed normal distribution, ranging from 83.83 to 84.10 µm for the first operator and from 83.83 to 84.38 µm for the second operator.

As calculated by means of Bland-Altman plots (fig. 1, 2), CRs for average GC-IPL thickness were 2.1 and 2.2  $\mu$ m for the first and the second operator, respectively (table 3).

**Table 1.** Patient demographics and characteristics

Age, years Gender, M/F Eye, R/L Intraocular pressure, mm Hg Visual field mean deviation, dB Central corneal thickness, um	29.63±5.1 21/39 31/29 14.1±2.5 +0.5±0.9 537 3+22 5
Central corneal thickness, µm	537.3±22.5
OCT foveal thickness, µm	252.34±7.23

Data are presented as means  $\pm$  SD or ratios. M = Male; F = female; R = right; L = left.

Interobserver intrasession CCCs for the GC-IPL average were 0.98 (95% CI: 0.96–0.99) for the first session and 0.96 (95% CI: 0.94–0.97) for the second session (table 4).

Intraobserver intersession CCCs for the GC-IPL average were 0.98 (95% CI: 0.97–0.99) for the first session and 0.97 (95% CI: 0.94–0.98) for the second session (table 5).

Minimum GC-IPL thickness data showed normal distribution, ranging from 80.78 to 81.85  $\mu$ m for the first operator and from 81.22 to 82.13  $\mu$ m for the second operator.

As calculated by means of Bland-Altman plots, CRs for minimum GC-IPL thickness were 9.0 and 6.3  $\mu$ m for the first and the second operator, respectively (table 3).

For the 6 sectoral measurements, CRs ranged from 3.3 (superotemporal sector) to 7.5  $\mu$ m (inferior sector) and from 1.7 (inferotemporal sector) to 4.5  $\mu$ m (inferonasal sector) for the first and the second operator, respectively (table 3).

Interobserver intrasession and intraobserver intersession CCCs are shown in tables 4 and 5, respectively.

#### Discussion

HD-OCT has been widely used to image the retina and its various anatomic layers with high resolution and good reproducibility [1, 2, 15–17]. Recent advances in segmentation algorithms have further allowed detailed separation and demarcation of individual retinal layers [2]. The study of retinal GCL by means of OCT has gained increasing interest as the GCL has been hypothesized to be the early site of neurodegeneration in glaucoma, diabetic retinopathy and demyelinating diseases [2, 5, 7, 8, 18–24]. The GCC, which is the sum of RNFL, GCL and IPL at macular regions, has similar glaucoma-discriminating performance compared with RNFL thickness [5]. None-

**Table 2.** Mean of GC-IPL thickness measurements (μm)

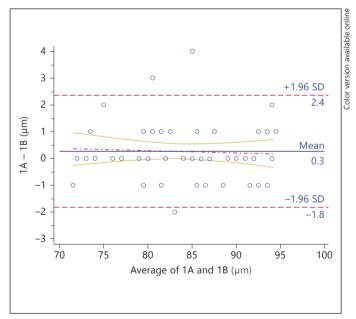
	Superior	Superotemporal	Superonasal	Inferior	Inferotemporal	Inferonasal	Average	Minimum
1A	85.13±1.75	82.52±1.71	85.18±1.78	83.73±1.85	84.07±1.95	83.95±1.90	84.10±1.25	81.50±1.45
1B	84.90±1.69	82.15±1.70	84.78±1.78	82.92±1.81	83.48±1.84	83.60±1.90	83.83±1.24	80.78±1.47
1C	85.27±1.73	82.07±1.70	85.85±1.77	83.25±1.80	83.43±1.85	84.18±1.88	83.98±1.25	81.85±1.44
2A	85.53±1.75	82.12±1.75	85.78±1.79	83.22±1.79	83.60±1.88	84.15±1.89	84.08±1.19	82.13±1.39
2B	85.25±1.65	82.12±1.70	85.35±1.75	83.27±1.79	83.63±1.80	83.60±1.86	83.83±1.20	81.22±1.40
2C	85.87±1.70	82.63±1.75	86.12±1.80	83.98±1.81	84.10±1.85	84.50±1.90	84.38±1.23	82.03±1.42

Data are presented as means  $\pm$  SD. 1A: measurements by observer 1 at the first session. 1B: repeated measurements by observer 1 at the same session. 1C: measurements by observer 2 at the first session. 2B: repeated measurements by observer 2 at the same session. 2C: measurements by observer 2 at the second session.

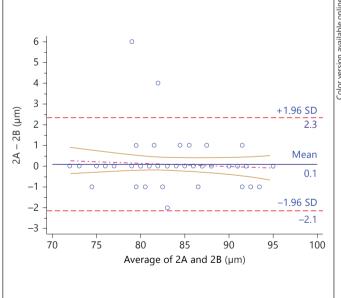
**Table 3.** Coefficients of repeatability of GC-IPL thickness measurements (μm)

	Average	Minimum	Superior	Superotemporal	Superonasal	Inferior	Inferotemporal	Inferonasal
CR (1A vs. 1B)	2.1	9.0	3.7	3.3	5.1	7.5	4.8	6.7
CR (2A vs. 2B)	2.2	6.3	4.1	2.5	4.4	3.3	1.7	4.5

1A: measurements of observer 1 at the first reading of the first session. 1B: measurements of observer 1 at the second reading of the first session. 2A: measurements of observer 2 at the first reading of the first session. 2B: measurements of observer 2 at the second reading of the first session.



**Fig. 1.** Bland-Altman plot showing intraobserver (observer 1) CRs for average RNFL thickness. 1A represents measurements by observer 1 during the first session; 1B represents repeated measurements by the same observer during the same session.



**Fig. 2.** Bland-Altman plot showing intraobserver (observer 2) CRs for average RNFL thickness. 2A represents measurements by observer 2 during the first session; 2B represents repeated measurements by the same observer during the same session.

Table 4. Interobserver intrasession reproducibility of GC-IPL thickness measurements

	Average	Minimum	Superior	Superotemporal	Superonasal	Inferior	Inferotemporal	Inferonasal
CCC (1A vs. 2A)	0.98	0.92	0.93	0.96	0.95	0.95	0.93	0.95
95% CI	0.96-0.99	0.89-0.95	0.88-0.95	0.93-0.97	0.92–0.96	0.92–0.97	0.89–0.95	0.93-0.97
CCC (1C vs. 2C)	0.96	0.91	0.93	0.96	0.93	0.93	0.97	0.95
95% CI	0.94-0.97	0.89-0.93	0.88-0.95	0.93-0.97	0.89–0.96	0.89–0.95	0.96–0.98	0.92-0.97

1A and 2A: measurements at the first session by observer 1 and 2, respectively. 1C and 2C: repeat measurements at the second session (30 days later) by observer 1 and 2, respectively.

Table 5. Intraobserver intersession reproducibility of GC-IPL thickness measurements

	Average	Minimum	Superior	Superotemporal	Superonasal	Inferior	Inferotemporal	Inferonasal
CCC (1A vs. 1C)	0.98	0.93	0.94	0.95	0.96	0.94	0.97	0.97
95% CI	0.97-0.99	0.90-0.95	0.91–0.96	0.91–0.96	0.93–0.97	0.91-0.96	0.95–0.98	0.96–0.98
CCC (2A vs. 2C)	0.97	0.92	0.95	0.94	0.96	0.95	0.97	0.96
95% CI	0.94-0.98	0.88-0.94	0.92–0.96	0.89–0.96	0.93–0.97	0.92-0.96	0.95–0.98	0.94–0.97

1A and 1C: measurements by observer 1 at the first session and at the second session (30 days later), respectively. 2A and 2C: measurements by observer 2 at the first session and at the second session (30 days later), respectively.

theless, it was questioned whether the inclusion of RNFL thickness in the GCC thickness measurement (from OCT devices such as Fourier-domain RTVue OCT; Optovue Corp., Fremont, Calif., USA) may have falsely elevated the diagnostic performance of the GCC [2, 12]. In contrast with this GCC measurement, the latest HD-OCT GCA algorithm (Cirrus HD-OCT software version 6.5; Carl Zeiss Meditec) can successfully demarcate the macular GC-IPL while excluding the RNFL.

Macular GC-IPL topography has been reported to be less variable among normal individuals than other diagnostically important structures such as the optic disc and RNFL [18], which may make the features of normal macular GC-IPL easier to identify and deviations from normal easier to detect. Recently, the ability of macular GC-IPL parameters to discriminate normal eyes and eyes with early glaucoma was found to be high and comparable to that of the best peripapillary RNFL and optic nerve head parameters [21].

van Dijk et al. [7, 8] reported a GCL thickness decrease in the macula as an early neurodegenerative effect on the retina of both types 1 and 2 diabetic patients with minimal diabetic retinopathy.

Macular GC-IPL thinning also occurs in multiple sclerosis eyes with and without optic neuropathy history, and GC-IPL thickness measurements may have better repro-

ducibility and superior structure-function correlations with vision than RNFL thickness measurements [20, 22, 23].

Ratchford et al. [24] demonstrated that multiple sclerosis patients with clinical and/or radiological nonocular disease activity, particularly early in the disease course, exhibit accelerated GC-IPL thinning.

The introduction into routine clinical use of new diagnostic technologies generates a question of the reproducibility and repeatability of measurements. Reproducibility refers to the closeness of agreement between independent results obtained with the same method on identical test material but under different conditions (different operators, apparatus, laboratories and time intervals). Repeatability is the variability of the measurements obtained by one person while measuring the same item repeatedly under the same condition.

Obviously, as with any new diagnostic technique, the reproducibility and repeatability of GC-IPL thickness measurements obtained with the Cirrus HD-OCT GCA algorithm is of prime clinical importance, being used for monitoring changes in neuroretinal damage independently from the underlying pathology.

In one of the first reports on the feasibility of thickness measurements of the RGC layer in glaucomatous eyes with an SD-OCT device (3D-OCT 1000; Topcon Inc., Tokyo, Japan), Wang et al. [19] manually performed their segmentation. This may lead to a higher intra- and interobserver variability than that obtainable from an automated system.

DeBuc et al. [1] developed an OCT retinal image analysis software (OCTRIMA) to be used with Stratus OCT and were able to detect and measure intraretinal layers, including the GC-IPL, in normal eyes.

Chiu et al. [25] tested an algorithm that accurately segmented the GC-IPL and 7 other retinal layers in normal eyes using a Bioptigen SD-OCT system (Bioptigen Inc., Research Triangle Park, N.C., USA), with a good agreement between observers and between manual and automatic segmentations.

Compared with the algorithms used by DeBuc et al. [1], Wang et al. [19] and Chiu et al. [25], the automated Cirrus HD-OCT GCA algorithm can segment the layers and measure 8 different parameters rather than just the average GC-IPL. This may facilitate objective comparison and selection of the most appropriate parameters.

In their study of automated detection and thickness reproducibility of GC-IPL with SD-OCT in glaucomatous patients, Mwanza et al. [2] used the macular cube  $200 \times 200$  scan protocol and a prototype algorithm (prerelease version), subsequently incorporated in the Cirrus 6.0 software, in 51 eyes of 51 glaucomatous patients. They used 5 scans per eye obtained on 5 days within 2 months and calculated the intervisit reproducibility of GC-IPL measurements using intraclass correlation coefficients (ICCs), coefficients of variation (CV), and test-retest SD (TRTSD). The highest reproducibility values were obtained for average GC-IPL and for superior parameters.

Takayama et al. [9] tested the glaucoma-discriminating ability of GC-IPL measurements using Cirrus HD-OCT and, contemporaneously, the intersession variability and reproducibility were assessed by means of ICC, CV and pooled within-subject TRTSD calculation. In normal eyes the best variability and reproducibility results were obtained for the average GC-IPL parameter (TRTSDS: 0.77  $\mu m$ , CV: 0.98%, ICC: 96%). Minimum and inferotemporal GC-IPL were less reproducible parameters.

In a recent study [10] assessing the reproducibility of thickness measurements of macular inner retinal layers using SD-OCT with or without correction of ocular rotation, an SD-OCT device (3D OCT-1000, version 2.13; Topcon, Inc.) was used to evaluate the intervisit reproducibility of several parameters, including GC-IPL, using the CV, reproducibility coefficient and ICC. The authors reported better and excellent reproducibility of the macular GC-IPL and GCC thickness compared with that of the

RNFL measurement in the macular and circumpapillary areas. In normal eyes, the CVs of the average GC-IPL thickness measurements ranged from 0.46 to 0.56%, while the reproducibility coefficients ranged from 1.35 to 1.64  $\mu$ m; ICCs ranged from 0.993 to 0.997.

In this observational case series study evaluating reproducibility and repeatability of GC-IPL thickness measurements using the most recent commercially available software version (6.5) of Cirrus HD-OCT in young healthy subjects, two experienced examiners independently performed 2 measurements in the first session and repeated the measurements in the second session 30 days later. Our results showed that the CR of average GC-IPL thickness measurements was excellent, resulting in 2.1 µm for the first operator and 2.2 µm for the second operator. Both interobserver intrasession and intraobserver intersession reproducibilities had very high results, with CCC values ranging from 0.91 to 0.99.

Our intersession results were as excellent as in previous studies [2, 9, 10]. However, none of these studies had assessed interobserver intrasession reproducibility, which may be of some interest when comparing clinical trials data. High interobserver intrasession reproducibility demonstrates the operator's ability to carry out a good examination, which depends not only on their own personal skill but also on their ability to use the instrument.

Technically, the high reproducibility may be due to the automatic detection and registration of the center of the macula using the algorithm included in the Cirrus software before placing the analysis annulus. Repeatability over extensive periods of time plays a particularly important role in the follow-up of patients with pathologies that have a slow evolution.

In our study, repeatability and reproducibility were slightly lower for minimum GC-IPL compared with average and sectoral GC-IPL.

A possible explanation of these results is the method of measurement of minimum GC-IPL. In fact, minimum GC-IPL is determined by sampling 360 spokes of measurements extending from the center of the fovea to the edge of the elliptical annulus in 1-degree intervals and selecting the spoke with the lowest average. Measuring GC-IPL on spokes may lead to lower reproducibility compared with measuring average and sectoral GC-IPL on a cube. In fact, minimum GC-IPL is calculated from only 50–60 sampling points in each B-scan compared with 7,500 sampling points in the macular elliptical annulus and 1,100–1,300 sampling points in the 6 sectors.

A limit to the present study is that the study population included only healthy young subjects. In our sample ex-

amination, time may be shorter than in older patients with neurodegenerative diseases. Further studies may be necessary to confirm this supposition and to test reproducibility and repeatability in older diseased patients.

In conclusion, the results presented in this study showed excellent reproducibility and repeatability of all 8 parameters in normal eyes, especially for average and sectoral, making the GC-IPL thickness an additional potential diagnostic marker for disease detection and progression in many pathologies involving the GCL. Further investigation will be needed to determine how the GC-IPL performs compared with both GCC and RNFL thicknesses.

#### **Disclosure Statement**

The authors state that they have no financial interest in any of the products mentioned in the study.

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