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Assessment of Axial Length Measurements in Mouse Eyes

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Abstract

Purpose—To compare measurements of murine ocular axial lengths (AL) made with 780 nm partial coherence interferometry (PCI) and 1310 nm spectral domain–optical coherence tomography (SD-OCT).

Methods—AL was measured at postnatal day (P) 58 in C57BL/6J mice. Repeated AL measurements were taken using a custom-made 780 nm PCI and a commercial 1310 nm SD-OCT. Intra- and inter-user variability was assessed along the central optical axis as well as two-degree off-axes angles with the SD-OCT. Data were collected and analyzed using Cronbach’s alpha (α), Bland-Altman coefficient of repeatability (CR), agreement plots, and intra-class correlation coefficients (ICC).

Results—Axial length measurements agreed well between the two instruments (3.262 ± 0.042 mm for PCI; 3.264 ± 0.047 mm for SD-OCT; $n = 20$ eyes). The ICC for PCI compared to SD-OCT was 0.92, confirming high agreement between the two instruments. Intra-user ICC for the PCI and SD-OCT were 0.814 and 0.995, respectively. Similarly, inter-user ICC for PCI and SD-OCT were 0.970 and 0.943, respectively. Using SD-OCT, a two-degree misalignment of the eye along the horizontal meridian produced mean differences in AL of -0.002 ± 0.017 mm relative to the centrally aligned images, while similar misalignment along the vertical meridian created 0.005 ± 0.018 mm differences in AL measurements.

Conclusions—AL measurements from the 780 nm PCI and 1310 nm SD-OCT correlate well. Multiple statistical indices indicate that both instruments have good precision and agreement for measuring murine ocular axial length *in vivo*. While the vertical meridian had the greater variability in AL in the small mouse eye; two-degree off-axes differences were within the standard deviation of centrally aligned AL.

Keywords

spectral domain-optical coherence tomography; partial coherence interferometry; low coherence interferometry; axial length; ocular biometry; myopia; refractive development

Myopia is a significant healthcare burden, affecting 41.5 % of the adult population in the United States¹ and greater than 80% of children aged 16–18 years in countries such as Taiwan² and Singapore^{3–4}. Most juvenile-onset myopia in children and induced myopia in animal studies have found that myopia is caused by excessive axial length that mismatches the optical power of the eye^{5–6}. Many studies suggest that myopia is caused by both genetic and environmental factors⁷. In order to elucidate the mechanisms underlying myopia,

further studies are needed in which both genes and visual input are manipulated. The mouse offers such a model where many genetic mutants are currently available and the visual environment can be easily altered^{8–12}.

While the mouse model offers many opportunities for myopia research, the small size of the eye, approximately 3 mm in diameter, represents a challenge in many aspects. Using a paraxial schematic eye model, Schmucker and Schaeffel have shown that a 5.4–6.5 μm change in axial length corresponds to a 1 diopter change in refractive error¹³. Thus, in order to monitor experimental myopia development in the various mutant mouse models, highly sensitive and noninvasive biometric techniques are needed for measuring axial length. Recently, a high resolution laser micrometer has been described with a resolution of 0.7679 μm ¹⁴. However, this micrometer can only be used on enucleated eyes. In this study we compare two *in vivo* methods for measuring mouse axial length: 780 nm partial coherence interferometry (PCI) and 1310 nm spectral domain-optical coherence tomography (SD-OCT). The PCI is a custom-built device¹⁵ and employs a principle that is similar to that of the commercial AC Master (Carl Zeiss Meditec AG, Jena, Germany). While the SD-OCT technology has been used with an advancing stepper motor to record axial length in mice¹¹, the 1310 nm SD-OCT used here offers the ability to visualize the entire globe in one image frame. We evaluated the agreement and precision of the two methods in measuring murine axial length by determining the Bland-Altman coefficient of repeatability (CR) and the intra-class correlation coefficient (ICC) as statistical indicators. In addition, we investigated the effect of two degrees of eye misalignment on axial length measurements using the SD-OCT. Our results indicate that both instruments offer good agreement in measuring AL in mice, and that relative to the central alignment of the eye with the SD-OCT, vertical misalignment created greater differences in measurements than horizontal misalignment of the eye.

METHODS

Animal Subjects

All animals were raised and treated in accordance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the institutional animal care and use committee (IACUC). C57BL/6J breeder pairs were ordered from the Jackson Laboratory (Bar Harbor, Maine, USA) and bred in Atlanta Veterans Affairs Medical Center Animal Facility. The mice were raised in 12/12 light-dark cycles (~10 lux) and provided chow and water ad libitum. Four independent C57BL/6J wild type litters for a total of 17 mice were measured at postnatal day 56–60 (P56–60; reported as P58). To determine agreement between instruments in measuring AL, measurements from each eye of an animal were treated as independent samples. The number of measurements that were analyzed for each eye is reported in the results.

Experiment Design and General Procedures

Partial Coherence Interferometry (PCI)—The PCI is based on the principle of a dual-beam Michelson interferometer with a partially coherent, near-infrared multimode diode light source that emits with a maximum emission at 780 nm and a coherence length of 120 μm . The output power is set at 175 μW . The movable mirror is adjusted such that the difference in the optical path length between the beams reflected from the movable mirror and stationary mirror in the interferometer matches the optical path length from the cornea to the retina to within the coherence length of the light source, at which point a concentric interference pattern is formed in front of the eye as shown on the display screen (Figure 1a). For a scan, the movable mirror is translated at a constant speed while the intensity of the interference pattern is measured by a photodetector and plotted as a function of the mirror position. Peaks in the resulting plot correspond to reflections from retinal surfaces (Figure

1b). The posterior peak of the eye was found to correspond to a reflection from the RPE-Bruch's membrane interface^{15–16}. Because the anterior corneal surface acts as a reference reflector in this setup, peak locations equal optical distances from the cornea. Thus, AL measured with PCI is defined as the distance from the anterior corneal surface to the RPE-Bruch's membrane.

For PCI measurements of axial length, we used a custom-built PCI¹⁵ that had been modified for the mouse eye. Each mouse in this study received a head pedestal at P28 as described previously¹⁷. At P58, mice were weighed and their eyes were dilated with 1% topical tropicamide (Bausch & Lomb Incorporated, Tampa, FL) to cause pupil dilation; mice have a small ciliary muscle that likely does not contribute to accommodation^{10, 18}. The awake mice were held in a plastic cylinder with the head immobilized by clipping the head pedestal to an adjustable stage. The optical axis of the mouse eye to be measured was carefully aligned along the collimated PCI measurement beam such that the 1st Purkinje image produced by the reflection from the anterior cornea was positioned in the center of the pupil (Figure 1a). On the same eye, two users performed two repeated trials where twenty scans were taken per trial. Not every scan produces a peak corresponding to a reflection from the RPE due to eye movement or deterioration of the tear film. At least ten scans that displayed RPE peaks were selected and averaged to determine the mean optical AL. Custom software converted optical AL to geometric AL through division by an average refractive index of 1.433¹⁶.

Spectral-Domain Optical Coherence Tomography (SD-OCT)—We used a 1310 nm SD-OCT (Bioptigen Inc, Durham, NC, USA) to measure AL. Similar to the PCI described above, OCT uses the principle of partial coherence interferometry to display the location of light-reflecting surfaces with respect to a reference mirror. Unlike PCI, where a scanning mirror matches the optical path length difference in the interferometer with optical distances between the cornea and retinal surfaces and a detector measures the intensity of the interference signal as a function of the mirror position, SD-OCT uses a stationary reference mirror, and a spectrometer measures the interference signal as a function of the wavelength of the reflected light reflected from ocular surfaces. Fourier transformation into time domain then produces the depth profile of ocular surfaces. A two-dimensional image is then created by combining a series of lateral depth scans (Figure 2a–c). This particular SD-OCT system has an axial resolution of 8 μm with a hardware-limited imaging depth of 4 mm and a loss-limited imaging depth of 1.5–3 mm (information provided by the manufacturer). Due to the size limitations of the instrument's image window, the 4 mm image was viewed by advancing the anterior segment such that it inverts and both the anterior and posterior segments become overlapped within the same window (Figure 2b). That is, the reference arm length was set to approximately the mid-position in the crystalline lens so that both “halves” of the eye appeared within the available range of the instrument, taking advantage of the “mirror artifact”^{19–20}. The ‘unfolded’ image was created using Adobe Photoshop to illustrate the true eye image (Figure 2c).

Mice were first measured awake with PCI and thereafter under anesthesia with SD-OCT using intraperitoneal injections of ketamine (80 mg/kg)/xylazine (16 mg/kg). The anesthetized mouse was placed in a heated cylindrical holder (36°C) which was attached to an X-Y-Z movable stage (Bioptigen Inc, Durham, NC, USA) in front of the SD-OCT light source. The cornea was hydrated with normal saline drops. The reference arm and focus dial were adjusted simultaneously to a point where all structures of the eye are in focus. Correct alignment was confirmed by viewing the radial image of the surface of the eye and adjusting the light source for the central reflection along the horizontal and vertical optical meridians (Figure 2a). Four to five linear scans were acquired per eye; each scan contained an average of 10 images. To measure AL, calipers (calibrated at refractive index of 1.433¹⁶) were

placed from the cornea to lens fold and RPE border to lens fold (Figure 2b). The sum of the two caliper measurements was recorded as the geometric AL. Again, at P58, inter and intra-user data were obtained by two users performing two trials per eye. Upon completion of imaging, the mouse was injected with yohimbine (2.1 mg/kg) to reverse the effects of xylazine and placed on a heating pad to recover.

Effects Of Eye Alignment on AL Measurements—To determine the influence of misalignment from the optical axis on AL measurements, an initially centrally aligned mouse eye was intentionally misaligned using degree markings on the SD-OCT animal rotary stage. The eye was misaligned by 2 degrees either along the horizontal or vertical meridian, with the mouse repositioned, centrally aligned, and then misaligned for each measurement. Misalignment greater than 2 degrees was not possible since the central cornea was no longer visible in the image to measure axial length accurately. Images (n=6 eyes) were acquired and analyzed as described above.

Statistical Analysis

Collected data were organized into agreement and Bland-Altman plots²¹. They were analyzed for precision and validity by using two statistical indices: 1) Bland-Altman CR for precision and 2) ICC for validity of same measure. Two other commonly used statistical indices were used for a familiar comparison: 1) Cronbach's alpha (α) for internal consistency and 2) Pearson's correlation coefficient (PCC) for agreement. All data analysis was performed by commercial software (PASW, ver 18.0; IBM SPSS, Somers, NY). All measurement values were reported in mean $\pm$ standard deviation.

RESULTS

Variability, Repeatability and Reliability of PCI

The instrument variability of the PCI was calculated by using the standard deviation of repeated measurements in one eye. Average standard deviation in AL measurements was 0.021 ± 0.045 mm for C57BL/6J mice at P58 (n>20 PCI scans/eye in 14 eyes of 7 mice). Figure 3a–d summarizes the data agreement plots and Bland-Altman plots for intra-user and inter-user AL measurements using the PCI. The reliability (accuracy) of intra-user AL measurements as determined by the ICC was 0.814 (n > 20 PCI scans/eye in 14 eyes) for the PCI. Statistical analysis of inter-user AL measurements showed a high ICC of 0.97 (n > 10 PCI scans/eye/user in 20 eyes). Other statistical parameters can be found in Table 1.

Variability, Repeatability and Reliability of SD-OCT

The instrument variability of the SD-OCT was calculated by using the standard deviation of repeated measurements in one eye. Average standard deviation in AL measurements was 0.010 ± 0.010 mm for C57BL/6J mice at P58 (n>8 scans/eye in 16 eyes of 8 mice). Figure 4a–b presents the agreement and Bland-Altman identity plots for intra-user AL measurements using the SD-OCT with realignment and focus adjustments of the mouse eye preceding each measurement. The reliability (accuracy) of intra-user AL measurements as represented by the ICC was 0.995 (n > 8 scans/eye in 16 eyes) for the SD-OCT. Figure 4c–d shows the agreement and Bland-Altman identity plots for inter-user AL measurements using the SD-OCT. ICC for inter-user reliability of AL measurements with realignment of the mouse between each successive image was 0.943 (n > 4 scans/eye/user in 19 eyes). Table 1 reports CR and Cronbach's alpha for internal consistency.

Agreement and Precision of PCI and SD-OCT AL Measurements

Average axial length measurements made on four separate litters were 3.262 ± 0.042 mm for PCI and 3.264 ± 0.047 mm for SD-OCT ($n = 20$ eyes). Figures 5a and b show the corresponding agreement and Bland-Altman identity plots for PCI and OCT murine ocular axial length measurements. The two instruments had an ICC of 0.920 and CR of 0.048 mm. Additional statistical parameters can be found in Table 1.

Effects of Misalignment

Figure 6 illustrates the effects of horizontal and vertical misalignment of the eye on AL measurements using the SD-OCT. While there were no significant differences between the measurements obtained in the four regions, the mean difference in AL was greater in the vertical meridian (0.005 ± 0.018 mm) compared to the horizontal meridian (-0.002 ± 0.017 mm). As shown in Figure 6, these differences were within the variability of the SD-OCT (0.010).

DISCUSSION

Methods for Ocular Biometry

Currently, the ocular biometric methods that have been tested and reported in the literature include A-scan ultrasonography^{13, 22}, calipers and micrometers¹⁴, image analysis of histological sections²³, MRI and CT scans²⁴, PCI (also called optical low coherence interferometry (OLCI)¹⁵, and more recently, SD-OCT¹¹. Techniques performed *in vitro* or *ex-vivo* (calipers, micrometers, histological sections) are associated with changes in eye length secondary to desiccation and loss of turgidity which compromise their reliability in detecting and measuring small changes in axial length (see¹⁴). There is also limited resolution of these techniques to detect small changes in AL, although the use of a laser micrometer has somewhat circumvented some of these issues¹⁴. With an axial resolution of about 40–50 μm , A-scan ultrasonography lacks the needed sensitivity for the small mouse eye in which a 10 D change in refractive error would be needed in order to detect a change in AL¹³. Coherence interferometric techniques (OLCI and PCI) have the advantage of being able to image the mouse eye *in vivo*, however, the informational yield has been mainly limited to axial parameters: AL, corneal thickness and anterior chamber depth¹⁶. A 1310 nm SD-OCT with stepper motor focal plane advancement has been reported to measure various structures within the mouse eye¹¹, but does not produce a cross-sectional image, as seen in Figure 2.

In vivo Ocular Measurements

The objective of this study was to determine the agreement and sensitivity of two instruments used to measure *in vivo* AL in the small mouse eye. Although *in vivo* mouse ocular AL has been reported previously^{11, 16}, a comparison of methods has not been completed. As previously mentioned in the results, the AL were 3.262 ± 0.042 mm for PCI and 3.264 ± 0.047 mm for SD-OCT. These values are within the standard deviations of previously reported values during similar time points^{11, 16}. The low Bland Altman CR values (0.079 mm PCI and 0.018 mm for SD-OCT) indicate that the instruments have good reproducibility. Visually, the scatter shown in Bland-Altman identity plots of each instrument (Figure 3 and 4) suggest no specific trend or cluster differences between two instruments. The Bland-Altman plots indicated small mean differences of 0.003 to 0.038 mm between instruments, users, and trials. These two factors provide additional evidence that the AL measurements does not depend on any systemic errors from the instruments. The ICC values for inter- (>0.943) and intra-users (>0.814) indicate strong correlations. These comparisons were further strengthened by calculating the Cronbach's alpha for internal

consistency which suggest high internal consistencies not only between trials, but also between independent users.

Compared to PCI recordings from other species¹⁵, the RPE/choroid peak in the mouse was relatively small and could be missed if not averaged. In the SD-OCT, some imprecision was generated by the subjective placing of the caliper on the RPE/choroid border which appeared as a bright band (Figure 2). However, even with these inconsistencies, the instruments still had precision of 0.010 ± 0.010 mm; corresponding to approximately a 2–4 diopter shift in refractive error.

Off-axis Measurements

One of the challenges in using the mouse eye is the absence of the fovea to align along the visual axis. Thus, alignment on the optical axis is based on visualization of Purkinje images¹⁵. In this study, we showed that misalignment in the vertical meridian produced the greatest change in AL. However, these changes were not greater than the standard deviation for centrally aligned instruments. Using the SD-OCT, we were only able to measure off-axis 2 degrees in each direction. The trend in shorter AL measurements in the nasal direction may suggest that the AL of the mouse eye is not uniform around the optic axis, particularly if measured beyond 2 degrees. Such regional differences in AL would be similar to those reported for primates, guinea pigs, and children.^{25–26} These results also indicate that greater care needs to be taken when aligning the mouse eye along the vertical versus horizontal meridian when performing any optical based technique such as photorefractometry, PCI, or OCT.

CONCLUSIONS

In conclusion, these results support the use of both the PCI and SD-OCT for measuring AL in the mouse eye. The PCI has advantages in speed of aligning the instruments and collecting data while the OCT offers the ability to image several ocular structures along with AL. In addition, we were able to show that misalignments <2 degrees from the central optical axis will not affect AL measurements in the mouse eye.

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Figure 1.

PCI alignment (**A**) and AL recording (**B**) from a C57BL/6J mouse at 58 days of age. (**A**) The PCI was aligned by positioning the Purkinje image (white arrow) in the center of the pupil. The nucleus of the lens was often visible and could also be aligned. (**B**) The PCI trace produced a larger peak at the RPE/choroid interface (arrow). The measurements are optical length as referenced from the anterior corneal surface. The values were converted to geometrical length by dividing by the refractive index of 1.433, as used previously.¹⁶

Figure 2.

Murine ocular biometry as measured with a 1310 nm SD-OCT. **(A)** The eye was aligned by positioning the Purkinje image in the center of the pupil. **(B)** A typical SD-OCT image in which the anterior and posterior portions of the eye are superimposed. AL is calculated by combining the caliper distance from the lens fold to anterior corneal surface and from the lens fold to the RPE/choroid interface. **(C)** An “unfolded” SD-OCT image created in an imaging program to better visualize the mouse eye in cross-section.

Figure 3.

Agreement (**A and C**) and Bland-Altman plots (**B and D**) for the PCI of AL in 58 day old C57BL/6J mice. (**A and B**) Intra-user data in which the same user took repeated measurements on the same mice. (**C and D**) Inter-user data compared measurements from two independent users for the same mouse eyes. The diagonal line (**A and C**) indicates a perfect 1:1 relationship.

Figure 4.

Agreement (**A and C**) and Bland-Altman (**B and D**) plots showing AL measurements taken with the 1310 nm SD-OCT. Intra-(**A and B**) and inter-(**C and D**) user data is shown for 58 day old C57BL/6 mice. The diagonal line (**A and C**) indicates a perfect 1:1 relationship.

Figure 5. Agreement (A) and Bland-Altman (B) plots comparing AL measured with the PCI and 1310 nm SD-OCT in 58 day old C57BL/6J mice.

Figure 6.

Box plot of differences between central axis and 2° off-axis AL measurements recorded with the 1310 nm SD-OCT. Measurements were taken in the superior, nasal, temporal and inferior directions in 58 day old C57BL/6J mice. Off-axis measurements had the greatest variability in vertical meridian relative to centrally aligned images. The upper and lower quartiles (25%) are shown by the box with a median line. Lines extending from top and bottom of the box indicate the maximum and minimum values. The individual points are outliers that are greater than 1.5 X the length of the box (interquartile distance). Dotted lines indicate the variability of the SD-OCT on centrally aligned images.

Table 1

Statistical comparisons of axial length between the PCI and SD-OCT in 58 day old C57BL/6J mice.

Measurements	CR	Cronbach's α	PCC	ICC
PCI intra-user	0.079	0.814	0.730	0.814
PCI inter-user	0.023	0.970	0.942	0.970
OCT intra-user	0.018	0.995	0.990	0.995
OCT inter-user	0.046	0.944	0.894	0.943
PCI vs OCT	0.048	0.920	0.855	0.920

CR: Bland-Altman coefficient of repeatability (mm); Cronbach's α for internal consistency; PCC: Pearson's correlation coefficient for agreement; ICC: Intra-class correlation coefficient for accuracy.