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Using Vascular Landmarks to Orient 3D Optical Coherence Tomography Images of the Mouse Eye

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Abstract

Comparing 3D structural information obtained by optical coherence tomography (OCT) requires accurate alignment of images acquired from individual subjects. Despite the widespread use of OCT to image the anterior and posterior mouse eye, few approaches to align the resulting image data have been described, in part due to a lack of well-characterized landmarks that are suitable for alignment. Here, we provide an OCT acquisition and analysis protocol that incorporates the use of the long posterior ciliary arteries as landmarks. In mammals, these two large choroidal vessels lie in a plane approximately parallel to the horizon. Our OCT imaging approach resolves these vessels in the mouse eye and suggests that their location is reproducible. The protocol may be useful for preparing 3D OCT data to compare experimental cohorts of mice and for standardizing results from independent research laboratories.

Keywords

mouse models; noninvasive imaging; optical coherence tomography; Fiji/ImageJ analysis; atlas landmarks

INTRODUCTION

Commercially-available OCT instruments provide an important non-invasive tool for assessing ocular tissue morphology in healthy and diseased mice. Most commercial instruments employ spectral-domain OCT, in which a near-infrared coherent beam of light is divided into one path that is reflected by a mirror and a second path that is reflected by a tissue of interest, such as the anterior or posterior eye of an anesthetized mouse. Due to differences in the refractive indices of the various cellular layers that comprise the tissue, the light reflected from the tissue is delayed, resulting in a unique spectral interference pattern when combined with light reflected from the mirror. This interference pattern is processed mathematically to generate a 1D representation of the tissue layers (A-scan) at the location where the sample beam is aimed. As the beam is swept back and forth, it produces 2D images (B-scans) that mimic histological cross-sections; when these sweeps are advanced orthogonally in raster fashion, a 3D dataset is generated. B-scans and 3D OCT images of the anterior and posterior mouse eye are analyzed to assess key morphological parameters (corneal and retinal layer thicknesses, anterior and posterior chamber depths) and to reveal

changes in tissue morphology, such as those that arise during development or from injury and disease.

To compare OCT data reproducibly between individuals, image datasets must conform to a standard geometry. Typically, the optical path of the OCT instrument is aimed at the optic nerve head, which lies near the center of the posterior eye. This produces *en face* images of the central retina that can be compared in principle because they are centered at the same point. However, this approach assumes that rotation around the optical path is identical in all subjects. Although this assumption is approximately correct, variation in head position may introduce rotational variation across samples and may contribute to differences in data from independent researchers and laboratories. Thus, while broad comparison of regional features is currently possible, a more reliable and standardized approach is needed to examine the distribution of fine morphological details in the healthy or diseased eye, such as localized changes in retinal thickness or a regional distribution of disease-associated lesions. The issue of rotational orientation around the optical path applies to other ocular tissues, such as the cornea, and to other noninvasive imaging methods, such as bright field fundus imaging and confocal scanning laser ophthalmoscopy cSLO.

This protocol describes the use of the two long posterior ciliary arteries (LPCAs) as internal landmarks for rotational orientation of 3D OCT datasets. In humans, the LPCAs lie roughly in a plane parallel to the horizon and serve to mark the horizontal meridian (Zinn, 1988). The nasal (medial) and temporal (lateral) LPCAs originate in the central ophthalmic artery, enter the choroid through the sclera on either side of the optic nerve head and continues to the ciliary margin, where they bifurcate to encircle the iris (Kiel, 2010). These large vessels can be distinguished from other choroidal arteries because they exhibit limited branching and relatively few bends or turns. Although they are often not noted explicitly, the LPCAs have been detected in mouse eyes by many *ex vivo* techniques. In a striking example, two large terminally branched vessels extending from the optic nerve to the periphery are revealed by optical projection tomography of autofluorescent tissue in nonpigmented mouse eyes (Carpanini et al., 2014). The bright “horizontal stripe” in pigmented eyecups used routinely by neurophysiologists to mark the division between the dorsal and ventral retina (Wei, Elstrott, & Feller, 2010) probably corresponds to the LPCAs. The LPCAs have also been observed in corrosion casts of the ocular vasculature (Ninomiya & Inomata, 2006) and are prominent in micrographs of mouse retinal pigment epithelium (RPE) flatmounts stained with fluorescently-tagged phalloidin (Collin et al., 2015), which binds to F-actin in the arterial walls.

Recently, we presented images identifying a portion of the LPCAs in 3D OCT datasets of the mouse posterior eye centered on the optic nerve head (Krebs, Xiao, Sheppard, Hicks, & Nishina, 2016). We proposed that these vessels might be useful as rotational landmarks. In support of this idea, we find that the location of the LPCAs relative to the optic nerve head is remarkably consistent among a large number of OCT datasets collected over >5 years (unpublished results). In addition to standardizing image rotation for the analysis of posterior ocular tissues, the LPCAs may serve in principle as rotational landmarks for anterior tissues, such as the cornea or iris, and may be applied to orient data obtained by other noninvasive imaging techniques.

STRATEGIC PLANNING (optional)

This protocol requires a commercial or custom OCT instrument capable of acquiring 3D data from the mouse eye with adequate resolution and depth penetration to detect major choroidal blood vessels. The instrument must also capture a sufficiently large area of the central posterior eye to include a portion of both LPCAs. In developing this protocol, the authors used a Bioptigen OCT instrument (Leica Microsystems). Devices with OCT capabilities are also available from Phoenix Research Laboratories, Heidelberg Engineering, Wasatch Photonics among others, but we have not tested whether any of these instruments can detect the long posterior ciliary arteries in mice. It may be possible to adapt aspects of the protocol described below for use with these instruments.

OCT instruments are typically supplied with excellent software packages for data acquisition and image processing. However, to make use of additional functionality that is unavailable in the manufacturer's software, researchers may choose to export image data for processing and analysis with third-party software. For example, the freeware Fiji (Schindelin et al., 2012), a bundled version of ImageJ2 (Schindelin, Rueden, Hiner, & Eliceiri, 2015), may be used to align, average and analyze 3D OCT datasets (Krebs et al., 2016). This program provides a large suite of image processing algorithms as well as the possibility of macro and plugin development tailored for image data analysis. A final strategic consideration is the amount of raw data generated by 3D OCT analysis that can be voluminous, rapidly filling terabyte drives. Adequate computer or server capacity is needed for data storage and backup.

BASIC PROTOCOL 1. 3D OCT of the Posterior Mouse Eye

Introductory paragraph

The fundamental approach is to acquire a OCT volume dataset that resolves the positions of the LPCAs on either side of the optic nerve head. Enhanced depth imaging (EDI) is used to yield datasets in which the LPCAs are easily visualized. The Support Protocols provide examples of how the positions of the LPCAs can be used to standardize image analysis so that commonly measured parameters, such as retinal layer thickness, can be compared reliably among individual mice and experimental cohorts.

Materials

Mice to be studied. Mouse strains must be bred and maintained with institutional animal care and use approval.

Gloves for handling mice

1% atropine sulfate ophthalmic Solution drops (Akorn, Lake Forest, IL)

Ketamine/xylazine cocktail for anesthesia [1.6 ml ketamine (100 mg/ml), 1.6 ml xylazine (20 mg/ml) and 6.8 ml sodium chloride (0.9% w/v)]

1 ml syringes and 26 g needles for injecting anesthesia

GenTeal Severe Dry Eye Relief (Alcon Laboratories, Fort Worth, TX)

Systane Ultra Lubricant Eye Drops (Alcon Laboratories, Fort Worth, TX)

Sugi surgical spears (Kettenbach LP, Huntington Beach, CA)

Bioptigen Envisu R2210 spectral domain OCT imaging system (Leica Microsystems GmbH, Wetzlar, Germany)

Personal computer (Mac OS X or Windows-based) with good graphics capability, 64-bit

Installed Fiji freeware, accepting all updates except developer tools on installation

Protocol steps—We use a Bioptigen ultrahigh resolution (UHR) Envisu R2210 Spectral Domain OCT Ophthalmic Imaging System (Leica Microsystems), with InVivoVue 1.4.0.4260 software installed on a 32-bit Windows computer as supplied by the manufacturer. A more recent 64-bit version of the software is available (contact Leica Microsystems). It may be possible to adapt this protocol to other instruments by using the manufacturer's software or by exporting data in *.tif format and using third-party software for analysis (see Support Protocol 3).

1. Turn on the instrument, the computer and the OCT light source.
2. Start the InVivoVue software.
3. In the Add Patient window, enter mouse-specific information for all mice to be examined in order during a single session. For simplicity, enter a unique mouse identifier into the first field (ID) and then copy and paste it into the two remaining fields (First, Last). Sex and date of birth must also be provided (Figure 1).
4. Create a custom rectangular volume scan that can be applied to each mouse examined. To move to the scan setup page, select the first mouse in the patient window, select Add Exam and then Start Exam. In the Add Patient Scans window (Figure 2), select Add Custom Scan and set up a Rectangular Volume scan with the following parameters: Length, 1.5 mm; Width, 1.5 mm; Angle, 0 degrees; Horizontal Offset, 0 mm; Vertical Offset, 0 mm; A-Scans/B-Scan, 1000 lines; B-Scans, 100 lines; Frames/B-Scan, 1 frames; Inactive A-Scans/B-Scan, 80 lines. Typically the right eye is examined first, so under Oculus Selection check "OD". If both eyes are to be examined routinely, add a scan and repeat these parameters with "OS" selected. Under Add Patient Scans, Define Protocol, enter a name describing the protocol containing the custom scans you created and Save Protocol. In subsequent sessions, this scan protocol can be loaded from a drop-down list of available protocols by selecting "Add-Defined Order".
5. Dilate the eyes of all mice to be examined. Wearing gloves, scruff each mouse in one hand and apply a drop of atropine sulfate directly onto each eye. Dilation should occur within 5–10 min and remain effective for an hour or more. Whiskers (vibrissae) may be trimmed to prevent them from interfering with imaging, but with practice they may be displaced by the red aiming tip at the end of the probe (See Step 11 below), so this is not critical.

6. Align the nodal point of the instrument with the metal phantom provided by the manufacturer.
7. Record the weight of mice and anesthetize one mouse with 0.10 ml of the ketamine/xylazine cocktail per 20 g body weight using a syringe and 32 g needle. The animal is considered anesthetized if no sudden movement is noted when firm pressure is applied to the hind limb. Sedation requires several minutes.
8. Apply a drop of GenTeal Severe gel to both eyes of the sedated mouse. This will prevent the corneal surface from drying prior to image acquisition.
9. When ready to image, dilute the GenTeal Severe gel by applying a drop of Systane Ultra to the eye being imaged. Remove excess liquid by touching a tissue to the corner of the eye, avoiding the corneal surface. Repeat this rinse twice. Leave a small amount of liquid on the ocular surface to moisten it during alignment with the optical path.
10. Place the mouse in the positioning stand (AIM-RAS in Bioptigen nomenclature) so that the eye to be examined is closest to the probe. Use the bite bar and dental cotton rolls or tissue on either side of the body to secure the mouse within the stand. For small mice (10 g or less), extra padding under the lower jaw may be needed to position the head and eye properly. Secure the Velcro strap provided by the manufacturer over the back of the mouse to hold the mouse and padding in position. (Figure 3A)
11. Align the eye with the probe optical path. Place the red aiming tip on the end of the probe. The tip may be left on the probe for the entire session to help with positioning. Turn the crank on the sample arm to advance the probe head within several millimeters of the corneal surface. To position the mouse, twist the white cylindrical tube of the mouse stand and rotate the stand around the vertical axis until the eye is close to the center of the red aiming tip (Figure 3A, B).
12. Select Start Aiming to initiate live imaging so that the retinal position can be adjusted. Use the reference and sample arm cranks to position the probe head so that the choroid is nearest to the top of the left-hand display, positioned roughly 1/5th of the total vertical distance from the top. The retinal image should be horizontal in the left-hand live display and vertical in the right, the optic nerve head centered and the retinal layers relatively free of curvature. To achieve these results, Bioptigen recommends using the bite bar adjustment knobs. We prefer twisting the white cylindrical tube to center the image of the optical nerve head in the vertical display coupled with adjustment of the stand height and lateral position (Figure 3A). If adjustments are made in this way, the eye may be displaced with respect to the nodal point, but this is less critical if imaging of most subjects is centered on the optic nerve head, as in our work.
13. Once the eye is aligned, remove excess liquid by wiping it gently from the ocular surface with a surgical spear. This step is essential for obtaining the highest image quality, as excess liquid causes blurring of the OCT image. A slight realignment may be required if the eye has moved during this procedure.

14. Immediately start the rectangular volume scan by selecting Start Aiming and then Snapshot. The scan completes in roughly 3 sec. Select Save Scan to save the data.
15. To view the LPCAs, use the windowing display tools on the Volume Intensity Projection (VIP) window of the InVivoVue software. Reduce the height of the calipers and position them below the choroidal reflections by moving the position slider. With appropriate adjustment, two bright, nearly linear reflections that point from the perimeter of the scan area toward the optic nerve head will be visible (Figure 4). These reflections correspond to the scleral vessel walls of the two LPCAs.
16. Remove the mouse from the stand and repeat steps 5–14 with additional mice as needed.
17. At the end of the session, exit the software, shut down the computer and turn off the system. Files saved during the session can be loaded from within the InVivoVue software or by migrating to the Bioptigen image folder (usually within the Bioptigen\Data\images\site* folder on the D: drive).

Step annotations: Note 3. Mouse identifiers should be limited to a length of 10 character if the OCT Volume Averager plugin will be used (see Support Protocol 3).

Note 4. Memory capacity on a 32-bit Windows platform will limit the maximum size of a rectangular volume scan. The dimensions of 1000 A-scans per B-scan $\times$ 100 B-scans by 1 frame lie below this limit (abbreviated as 1000 $\times$ 100 $\times$ 1; frame refers to the number of repeat B-scans at the same raster position). The nonisotropic approach (the difference in the number of A-scans per B-scan versus the number of B-scans) allows high resolution in at least one dimension, and is therefore preferred for surveying fundus features, as in the protocol presented here. Newer Bioptigen 64-bit installations can acquire isotropic scans of 1000 $\times$ 1000 $\times$ 1.

Note 5. Prevent atropine drops from entering the mouth of the mouse, as this drug may be toxic if a large amount is ingested accidentally. If the mouse eye was treated with atropine or another dilating agent on the same day prior to OCT, additional treatment may be unnecessary.

Note 9. Proper hydration and removing excess corneal liquid are THE most important steps to ensure high quality images. If imaging takes more than a few minutes, reapply a drop of Systane and wipe it away with a surgical spear to keep the corneal surface moist.

Note 12. Some adjustment may be required to yield the sharpest images. A key parameter to optimize is image dispersion under the Resampling tab, which can be accessed through Tools>Configure Processing. In the scan window, initiate continuous scanning with Start Free Run and click on Optimize Frame in the Image Processing and Resampling Controls window as the continuous scan passes through the center of the VIP. The dispersion coefficients will be recalculated quickly and the image should appear sharper. In our experience, this adjustment may be necessary each session. For optimal calculation of

dispersion coefficients, Bioptigen recommends performing the same adjustment on a smaller scan with a width of 0.8 mm, selecting a flat region of the retina that can be positioned horizontally in the B-scan display, and expanding the vertical display with the Start and End sliders under the Options tab of the Edit Display Options window to assess the image quality.

Note 14. If the circular scan extends beyond the rectangular frame or are not centered on the frame, it may be necessary to adjust the Width, Length, Horizontal and Vertical offset in the Set Parameters for New Scan window. Several rounds of adjustment, scanning, and reviewing the display are typically required. Once adjusted, the settings should not need to be changed in subsequent sessions.

SUPPORT PROTOCOL 1 (optional). Measuring Retinal Thicknesses Following Rotational Correction

Introductory paragraph

This support protocol describes how the location of the LPCAs can be used to guide the selection of positions for measuring retinal layer thickness from OCT image data with digital calipers. The protocol may be implemented when only a limited number of measurements are anticipated or when more detailed analysis will not be pursued, such as the generation of retinal thickness heatmaps using Diver software (Bioptigen). This support protocol may also be used to assess thickness in previously collected datasets where the LPCAs are not horizontally aligned.

Materials

None

Protocol steps

1. Load an OCT rectangular volume scan acquired as described in the Basic Protocol.
2. In the VIP display, activate Windowing Display On and decrease the caliper height to the lowest tick mark. Position the center of the caliper sclera to the choroid so that the LPCAs can be visualized. Adjust the caliper height and position to optimize the appearance of the LPCAs in the VIP display (Figure 5A).
3. Right-click on the VIP display and select Caliper 01. Place the bottom end of this caliper at the site position it to span from the edge of one LPCA to the other (Figure 5B). Note the angle.
4. Decide on a distance from the optic nerve head where retinal layer thickness will be routinely assayed. For example, a distance of 300 μm from the optic nerve head is often the first position for so-called “spider plots” of layer thickness.
5. Reposition Caliper 01 by dragging on one end until its angle equals the angle noted in Step 3 plus 90 degrees and its length is twice the target distance chosen

in Step 4. Position this caliper so that its center lies at 0, 0 (that is, the image center). If acquisition was performed properly, the optic nerve head will be centered at this position (Figure 5B).

6. Using the Frame slider below the B-scan display, advance the frames until the line on the VIP display intersects one end of Caliper 01 on the VIP display (Figure 5B; either the superior or inferior end of the Caliper 01 may be chosen, but must be used consistently).
7. In the B-scan display, right-click and choose Caliper 01 (caliper numbering is independent for the B-scan and VIP windows). Right-click and choose Configure Calipers, migrate to the B-scan tab, and ensure that Caliper 01 is locked vertically so that measurements are axially oriented.
8. Return to the B-scan display. The lateral position of the caliper should now be apparent as a dot on the horizontal line in the VIP display. Drag the caliper over the B-scan and position it so that the dot lies at the intersection of lines in the VIP display. Now extend Caliper 01 in the B-scan display so that its ends mark the boundaries of the retinal layer to be measured (Figure 5C). Record the length given in mm to the right of the caliper. This length represents a measure of retinal layer thickness at a reproducible location with respect to the LPCAs.
9. Repeat Steps 1–8 for other datasets. The set of retinal layer measurements may be used for statistical analysis to detect differences among experimental cohorts.

Step annotations: None

SUPPORT PROTOCOL 2 (optional). Adjusting the Acquisition Angle to Align LPCAs Horizontally

Introductory paragraph

This support protocol describes how the location of the LPCAs can be used to reorient the scan angle during acquisition so that the LPCAs are aligned horizontally in the output dataset. The protocol requires extra time during acquisition to refine orientation, but it may be the only approach that enables downstream processing of oriented data by Diver software, at least in its currently available version. Thus, this protocol may be preferred when a more comprehensive analysis of focal and regional features is desired.

Materials

See Basic Protocol

Protocol steps

1. Follow the Basic Protocol through Step 14, in which the first volume scan is recorded from the subject. Do not save the data.
2. In the VIP display, check Windowing Display On and decrease the caliper height to the first tick mark. Position the center of the caliper sclera to the choroid so

that the LPCAs can be visualized. Adjust the caliper height and position to optimize the appearance of the LPCAs in the VIP display (Figure 5A).

3. Right-click on the VIP display, select Caliper 01, and position it to span from the edge of one LPCA to the other (Figure 5B). Note the angle.
4. Edit the selected scan by changing the Angle parameter to the angle determined in Step 3. This will result in a reorientation of the scan clockwise (positive angles) or counterclockwise (negative angles) so that the ends of the LPCAs visible in the scan area are connected by a horizontal line.
5. Return to live scanning, make any needed adjustments to retinal alignment, and proceed with scanning and saving as described in the Basic Protocol, Steps 12–15. With this approach, 3D OCT image data will be acquired in a standard geometry based on a horizontal orientation of the LPCAs.
6. The saved data may be accessed by using Diver software to perform more elaborate data analysis, including the generation of grid measurements, spidergrams or heatmaps of retinal layer thickness. The use of a standard geometry for such analyses will improve comparison of the topography of retinal features and pathology among experimental mouse cohorts.

Step annotations: None

SUPPORT PROTOCOL 3 (optional). Rotating 3D OCT Data for Analysis in ImageJ/Fiji

Introductory paragraph

This support protocol describes how to orient 3D OCT datasets by using third-party software, which may offer additional functionality not available in the packages provided by OCT instrument manufacturers.

Materials

None

Protocol steps

1. Run OCT Volume Averager on a multiple OCT files acquired as described previously (Krebs et al., 2016).
2. Use Fiji to review the *en face* volume generated as output by OCT Volume Averager. If parameters described in the Basic Protocol were used to acquire the OCT rectangular volumes, the output image stack will have xy dimensions of 1000×1000 with the number of slices corresponding to the number of pixels selected from the 1024-pixel axial depth as input when using OCT Volume Averager.
3. Use the line tool in Fiji to determine the angle of the LPCAs relative to horizontal (Figure 6A). This angle is indicated at the bottom of the Fiji menu bar.

4. Select Image>Transform>Rotate and enter the angle determined in Step 3. Select Apply to all slices in the image stack. The LPCAs should now appear horizontal (Figure 6B).
5. Proceed with further analysis as desired. For example, set the scale of the image under Analyze>Set Scale according to the acquisition parameters used in the Basic Protocol (Distance in pixels, 1000; Known distance, 1.5; Pixel aspect ratio, 1.0; Unit of length, mm). To create a smooth profile of the retina (Figure 6C), use Edit>Selection>Specify to enter the size and coordinates of a small circle at a selected location (for example, a 0.05 mm diameter circle 0.30 mm below the center in the ventral retina). Then create a z-axis profile at this point with Image>Stacks>Plot Z-axis Profile, and use the rectangle selection tool to generate a box on this plot that intersects with the midpoints of the gradients that approach hyperreflective peaks on either side of the outer nuclear layer (Figure 6C). The width of this box is indicated at the bottom of the Fiji menu bar, and may be saved to the Results window by using Analyze>Measure (first select Bounding rectangle and Display labels in Analyze>Set Measurement). This strategy can be repeated on many profile plots to create a table of data that can be copied to other programs.

Step annotations

- 1 The processing time for 10 replicate rectangular volume scans ($1000 \times 100 \times 1$) using the OCT Volume Averager plugin on a typical desktop computer is ~20 min, which create a *.tif image stack of B-scans and a *en face* view of a resized image.
- 5 The full capabilities of Fiji/ImageJ macro and plugin development can be used to expand and automate this process as desired. Several useful guidelines on how to create macros and plugins are available. Useful snippets of code can also be obtained by searching the internet for desired functionality (“how do I do x in ImageJ?”).

REAGENTS AND SOLUTIONS

None

COMMENTARY

Background Information

This technique arose from our interest in assessing the distribution of disease-associated lesions in mutant mouse models of human eye disease. Many lesions of the posterior eye are distributed nonuniformly. For example, dysplastic retinal folds and rosettes in *Crb1^{rd8}* mice were found by indirect ophthalmoscopy and noninvasive fundus imaging to be distributed mainly in the mouse nasoinferior retina (Luhmann et al., 2012; Mehalow et al., 2003). This result has been confirmed by microscopic examination of retinal and RPE whole mounts (unpublished results; (Luhmann et al., 2012). As a second example, dedifferentiated RPE

lesions in *Adams14^{trm267}* mice were localized by microscopy to the central retina with a slight inferior bias (Collin et al., 2015). Our awareness of the nonuniform distribution of ocular pathology in mouse models led us consider the importance of developing a standard geometry for analyzing data obtained by both noninvasive and microscopy-based imaging approaches.

Noninvasive images of the mouse eye are usually acquired without accurate orientation and are presented with the assumption that the top of each image corresponds to the dorsal (superior) eye. In one corrective approach, we attempted to account for orientation in brightfield fundus images by using an image of the external eye. Fundus images were rotated by an angle opposite to the angle of rotation of the edge of the nictitating membrane relative to the center of the pupil (Krebs et al., 2016; (Low et al., 2014), but this approach remains to be validated. We are unaware of methods to orient noninvasive images of the eye acquired from other commonly used non-primate research animals. However, comparative studies of retinal topography in fish species used images that were oriented with the assumption that the distribution of topographic features is similar among individuals (Cohn, Collin, Wainwright, & Schmitz, 2015). In human clinical studies, a related approach has been described to align and scale fundus images based on general features of the retinal vessel arcades (Abramoff, Garvin, & Sonka, 2010), which may find application as “atlas landmarks” that enable large comparative studies of fundus image data and automated detection of abnormal pathology. We note that although the precise position, shape and number of small blood vessels is unique in each individual, the location of large vessels such as the LPCAs may be sufficiently uniform to serve as rotational landmarks to enable similar comparative approaches.

We anticipate that noninvasive image analysis of the mouse eye by other methods, such as brightfield or fluorescent fundus imaging and cSLO, may benefit from the method described here. The images produced by these methods are typically centered on the optic nerve head without explicitly accounting for rotational orientation, which may be corrected by acquiring 3D OCT datasets in parallel from the same subjects. Registration and scaling of additional ocular features will be needed to align OCT datasets with the images obtained by these methods. For example, the superficial retinal vessels, which can be detected by both OCT and brightfield imaging or cSLO, may be useful for this purpose. Once aligned, image rotation based on the LPCAs would allow comparative studies of ocular features detected by brightfield fundus imaging or cSLO in a standard geometry.

Critical Parameters

In the current protocol, only short portions of the LPCAs near the optic nerve head are detected by the OCT imaging system used. By using a Bioptigen extra high resolution spectral domain OCT instrument capable of capturing an area of 1.8 mm × 1.8 mm, we have confirmed that these vessels extend further toward the periphery (unpublished results). However, future imaging studies at more nasal and temporal locations will be needed to determine whether the structure and position of these vessels is reproducible as they approach the ciliary margin, and thereby validate their usefulness as landmarks.

Although the OCT axial scale is reproducible, the lateral scale is influenced by changes in the thickness of tissues and the depth of fluid-filled chambers that lie in the optical path of the mouse eye. These changes mandate a reassessment of the default lateral scale as calculated by the manufacturer. For example, in hyperopic eyes, the posterior chamber depth may be decreased, yielding in a smaller area of capture for the OCT probe, which has a 50° field of view. As a result, the lateral scale ($\mu\text{m}/\text{pixel}$) is decreased. The current protocol does not correct for such deviations. If age-matched animals are used, the likelihood of differences in scale among individuals of the same strain is minimized. Furthermore, OCT can be used to assess changes in axial thicknesses or depths (Park et al., 2012) if they are suspected to occur in a mutant strain, so it may be possible to correct scaling for differences arising from these changes.

It may not be possible to use this protocol to study mouse mutants that affect vascular development, growth or maintenance if the structure or shape of the LPCAs is altered. In this case, it may be possible to use external ocular features as landmarks, such as the location of the center of the nasal nictitating membrane (Sterratt, Lyngholm, Willshaw, & Thompson, 2013). Additionally, independent studies are required to determine whether the external features are aligned reproducibly with the LPCAs in control mice.

Troubleshooting

One key to acquiring high quality data is to position the mouse properly so that the optic nerve head is centered, the live scans are horizontal and vertical in their respective display windows, and the overall curvature of the B-scan is minimized. The sharpest images are obtained by attentive removal of excess liquid, but care must be taken that the ocular surface does not become dry. In our hands, dispersion correction is imperative at least once a session. Excessive movement due to heartbeat and respiration will also interfere with good imaging. Removing the mouse from the stand for additional time in a warmed environment may help it to reach a more suitable level of anesthetization. If excessive movement is still observed, consider increasing the dose of anesthesia in future studies, which may vary among strains.

Anticipated Results

Use of this protocol is expected to improve the alignment among *en face* 3D OCT datasets of the mouse eye. The maximum deviation in the rotation of the LPCAs in datasets we have collected over >5 years approaches 40°, so correction for misalignment is anticipated to provide substantial benefit. We expect that rotational orientation based on the position of the LPCAs will improve the ability to detect small and subtle topographic features among healthy or diseased mouse cohorts.

Time Considerations

Basic Protocol. Positioning and imaging both eyes of a single mouse may require up to 10 min, although with practice this time may be reduced considerably. If 10 rectangular scans are to be obtained for use averaging (see Support Protocol 3), each mouse may require up to 15 min.

Support Protocol 1. Measurement of a single layer thickness from a saved image may require up to 5 min due to challenges in positioning the calipers precisely.

Support Protocol 2. Measurement of the angle of rotation needed to make the detected end of the LPCAs horizontal and updating the scan parameters to include this angle may require several minutes for each eye examined.

Support Protocol 3. Data processing with OCT Volume Averager as described in Support Protocol 3 for a single eye takes ~20 min for 10 rectangular volume scans (MacBook Air, 1.7 GHz Intel Core i7 processor, 8 GB 1600 MHz DDR3 memory), but good averaging results can also be obtained with as few as five scans, which require ~10 min to process.

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INTERNET RESOURCES

- <http://fiji.sc/Fiji> This site may be used to install the image analysis software Fiji.
- https://imagej.nih.gov/ij/docs/macro_reference_guide.pdf This site provides a manual describing how to program macros in ImageJ/Fiji.
- http://fiji.sc/List_of_update_sites This site may be used to install the OCT Volume Averager plugin from the Fiji update site. Updates may be added to any Fiji installation by launching the ImageJ updater with Help>Update Fiji, choosing Manage Update Sites, and selecting the JAX Eye update. Following the update, OCT Volume Averager will appear on the Plugin dropdown window of Fiji.
- <https://github.com/jaxcs/octvolavg> This site provides the source code corresponding to OCT Volume Averager available at GitHub. It is unnecessary to obtain this file unless further development of the plugin is desired.

The screenshot shows a software window titled "Add Patient" with a close button in the top right corner. Inside the window, there is a section labeled "Patient Details". This section contains several input fields: "ID" with the value "0047_1931", "First" with "0047_1931", "Last" with "0047_1931", and "MRN" which is empty. To the right of these fields are radio buttons for "M" (selected) and "F", and a date of birth (DOB) field showing "11 / 13 / 2016" with "MM", "DD", and "YYYY" labels below the respective boxes. Below the "Patient Details" section is a table-like structure for eye parameters. It has two columns, "OD" and "OS". Under "OD", there are two rows: "Refractive Error" with a value of "0.00" and "Axial Length" with a value of "23.0". Under "OS", there are two rows: "Refractive Error" with a value of "0.00" and "Axial Length" with a value of "23.0". Each value is in a box with up and down arrows. At the bottom of the window is a "Notes" text area, which is currently empty. Below the "Notes" area are two buttons: "Save & Exit" and "Cancel".

Figure 1.

The Add Patient dialog box in Bioptigen InVivoVue software. The same unique mouse designation may be used to fill required patient information boxes. Sex and date of birth (DOB) must be provided for each mouse but default values for remaining parameters may be kept.

Select Parameters for New Scan

Defined Scan Presets: [Dropdown]

Copy Settings from Selected Preset

Select Fixation Target: NONE

Select Parameters for Rectangular Volume

Annular Volume Linear B-Scan Mixed Volume Radial Volume **Rectangular Volume**

Length: 1.5 mm
Width: 1.5 mm
Angle: 0 degrees
Horizontal Offset: -0.02 mm
Vertical Offset: 0.02 mm

A-Scans / B-Scan: 1000 lines
B-Scans: 100 scans
Frames / B-Scan: 1 frames
Inactive A-Scans / B-Scan: 80 lines

Oculus Selection
☒ OD ☐ OS ☐ OU

Volumes
 Number of Volumes: 1 volumes

Doppler
☐ Doppler scan? Number of Intervals: [Dropdown]

New Scan Preset
 Enter Name: [Text Field] Create Preset:

System Memory
 16% of memory in use
 Scan requires 81.9% of remaining memory
 Scan Memory Requirements: 1562.5 MB

Add to Exam Cancel

Figure 2.

The Select Parameters for New Scan window of the BiopTigen InVivoVue software, showing settings for a custom Rectangular Volume scan. Key parameters include the number of A-scans per B-scan and the number of B-scans. The Length and Width of the acquisition area, as well as the Horizontal and Vertical offsets, may require empirical adjustment to ensure that the circular scan area is centered and just touches the edge of the scan window (see Basic Protocol, Note 14).

Figure 3. Positioning the mouse on the BiopTigen AIM-RAS stand for OCT imaging. A. Image showing the OCT probe bore (B), aiming tip (AT), mouse cassette (C), and bite bar (BB). The mouse position may be adjusted by twisting the cassette and by using knobs on the bite bar, the stand swivel (S) or the lateral adjustment (LA) and vertical adjustment (VA). B. Detail of A showing close approach of aiming tip to target eye. Note that proper alignment requires rotation of the mouse around more than one axis.

Figure 4. Scan display of BiopTigen InVivoVue software. The panel on the left shows the 50th B-scan of a rectangular volume scan ($1000 \times 100 \times 1, 1.5 \text{ mm} \times 1.5 \text{ mm}$) obtained from a C57BL/6J mouse at five months of age. The green horizontal lines indicate the caliper width and position used to generate the VIP shown in the panel on the right. The LPCAs appear in the VIP as hyperreflective structures (*arrowheads*) pointed toward the optic nerve head (*asterisk*). The green horizontal line in the VIP indicates the position of the B-scan in the left panel.

Figure 5.

Using the position of the LPCAs to standardize locations at which retinal thickness measurements are made. A. Display of a rectangular volume scan obtained from a retinal degeneration mutant model, optimized to emphasize the LPCAs. B. A caliper drawn to display the angle of the LPCAs (7.6°) relative to horizontal (0°). C. The right-hand panel shows a caliper orthogonal to that in B, adjusted to a length of 0.60 mm (that is, twice the target distance of 0.30 mm from the optic nerve head as selected for this example). This caliper is centered at 0, 0 and is thereby centered on the optic nerve head. The horizontal green line intersecting the end of the caliper indicates the position of the B-scan displayed in the left-hand panel. The position of the caliper in the left-hand panel is revealed by a dot along the horizontal green line of the right-hand panel. This position is adjusted so that the dot is located at the point where the green line intersects the 0.60 mm caliper. Total retinal thickness is then measured with a caliper in the left-hand panel, which is adjusted to span from Bruch's membrane to the vitreal retinal surface. The measured length of 0.165 mm is considerably lower than the C57BL/6J value of 0.215 mm, indicating degeneration.

Figure 6.

Rotational orientation and measurements using the LPCAs as landmarks in Fiji. A. Three *en face* images of the right eye of a STOCK *Crl1^{rd8}* mouse showing the retinal surface (*left*), outer nuclear layer (*middle*) and deep choroid (*right*). This dataset was obtained with a scan height and width setting of 1.4 mm, so the imaged area extends beyond the edges of the rectangular volume. Images were selected from replicate OCT rectangular volume scans processed by using OCT Volume Averager and displayed by using Image>Stacks>Tools>Grouped Z Project with a group size of 25. A line drawn in Fiji between the points at which the LPCAs intersect the circular image area gives an angle of 35.12° with respect to horizontal. B. The same images shown in A but rotated clockwise by 35.12°. White spots (dysplastic lesions) are observed to accumulate mainly in the ventral retina, but also extend to a horizontal boundary above the optic nerve head. C. Use of rotated data for retinal layer thickness measurements. 3D OCT volume scans of the right eye of a C57BL/6J mouse were acquired, processed and rotationally oriented in Fiji based on the LPCAs (*left*). A small circular area centered 0.30 mm ventral to the optic nerve head (*yellow circle*) was selected to generate a Z profile (*right*). Mean indicates mean image intensity (8-bit scale) and Slice indicates the position in steps along the z axis, which correspond to A-scan (axial) pixels. Arrows indicate the peak hyperreflectivity for retinal layers that flank the outer nuclear layer (OPL, outer plexiform layer; ELM, external limiting membrane). The red box drawn by using the rectangle selection tool in Fiji intersects with the midpoints of the

gradients that approach the OPL and EML peaks from the outer nuclear layer. The width of the box is 35.6 pixels, corresponding to a mean outer nuclear layer thickness of 54.3 μm within the selected circular area (the axial scale factor provided by the manufacturer for the instrument is 1562 $\mu\text{m}/1024$ pixels, or 1.525 $\mu\text{m}/\text{pixel}$).