Two-Photon Imaging of the Mouse Eye

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PURPOSE. To image the conventional aqueous outflow pathway and adjacent structures within the intact enucleated mouse eye using a noninvasive microscopy technique.

METHODS. Two-photon microscopy (2PM) techniques, including two-photon autofluorescence (2PAF) and second harmonic generation (SHG), were used to obtain images of the trabecular meshwork (TM) region within an intact mouse eye. Cardiac perfusion of fluorescein-conjugated dextran was used to label blood vessels within the eye to serve as an anatomic reference. Eyes were subsequently fixed, paraffin embedded, sectioned, and stained for comparison to the 2PM images.

RESULTS. Three-dimensional analyses of multiple 2PM images revealed a well-defined region adjacent to the iris and cornea that is free of SHG signal and consistent with the location of Schlemm’s canal. This open region is continuous with smaller tube structures consistent with collector channels. These structures do not label in mice perfused with the vascular probe dextran, supporting the hypothesis that the enclosed spaces are filled with aqueous humor rather than circulating blood. The TM region in the mouse eye was also visible, with a clear SHG signal representing collagen fibers.

CONCLUSIONS. These results support the hypothesis that 2PM may be useful for noninvasively imaging the conventional aqueous outflow pathway in mouse models of glaucoma. Studies are ongoing to validate our methodology in live animals. (Invest Ophthalmol Vis Sci. 2011;52:4098–4105) DOI:10.1167/iovs.10-7115

Glaucoma is a heterogeneous group of diseases that, as a group, is the second leading cause of blindness worldwide.¹,² Current diagnostic techniques for glaucoma, such as visual field testing and retinal nerve fiber layer measurements, lack disease specificity and provide little additional information beyond that of the common funduscopic examination. Currently, high intraocular pressure (IOP) is the only modifiable risk factor for glaucoma, although it is well known that many patients with glaucoma do not have elevated IOP.³ Furthermore, methods for measuring IOP are predisposed to inaccuracies because of operator error, corneal biomechanical differences, and other poorly understood variables.⁴ Finally, the measurement of IOP is merely a surrogate metric for the health state of the outflow system of the eye, and more direct structural and functional assessments of aqueous outflow would be desirable.

It is believed that structural abnormalities in the aqueous outflow pathway, especially in the juxtacanalicular trabecular meshwork (TM), the inner wall of Schlemm’s canal (SC), or both, may lead to elevated IOP and the development of glaucoma.⁵ Visualizing the TM region at high resolution would be of potential diagnostic value for the early detection of glaucoma and for following its progression in patients. Although currently available clinical imaging technologies such as optical coherence tomography (OCT) and ultrasound biomicroscopy (UBM) can image SC,⁶,⁷ they are unable to achieve high enough resolution to be of clinical value in most forms of glaucoma such as primary open angle glaucoma.

Two-photon microscopy (2PM) is a nonlinear imaging technology with subcellular resolution capabilities that has been previously used to image ocular tissues without the need for fixation.⁸–¹¹ 2PM involves two infrared photons interacting simultaneously with a target molecule. When 2PM is used to excite endogenous fluorophores it is called two-photon excitation autofluorescence (2PAF), and it is similar to traditional confocal fluorescence microscopy except that fluorescence excitation results from the absorption of two photons instead of one. 2PM can also be used to image by a process called second harmonic generation (SHG). SHG occurs when non-centrosymmetric macromolecular structures (principally collagen) simultaneously “scatter” instead of absorb the two infrared photons. This scattered energy resolves as a single higher energy (blue) photon of exactly half the excitation wavelength. The use of 2PM is significantly different in several ways from conventional microscopy (see Refs. 9, 12 for reviews). The excitation source is a high-intensity near-infrared laser with extremely short pulse duration (10⁻¹⁵ seconds). The near-infrared photons have greater tissue penetration than the higher energy photons used in confocal microscopy. The two-photon interaction occurs only where the objective focuses the laser, offering resolution similar in many ways to that of confocal microscopy. However, the duration of the 2PM laser pulse is so short that thermal damage should not occur in the tissue under the general conditions used for imaging. Additionally, the two-photon laser used for imaging is distinct from the two-photon laser used for surgical photodisruption of the TM,¹³ which is 10⁵ times more powerful.

Our group previously demonstrated the use of 2PM to image deeply into the TM of flat-mounted human cadaver tissue while in its native, unfixed state.⁹ In the present study, we evaluated the usefulness of 2PM for imaging the TM and nearby outflow structures within intact enucleated unfixed and fixed mouse eyes. Our findings provide the foundation for pursuing live animal ophthalmic imaging to further characterize the in vivo structure and function of the aqueous outflow system.

METHODS

Mouse Eye Tissue

Eyes were obtained as post-necropsy tissue from mice euthanatized at the conclusion of other unrelated research projects. Mice from a
C57BL/6 strain with no ocular phenotype were provided by Mark Petrasch (University of Colorado, Denver), and DBA/2J mice were provided by Moshe Levi (University of Colorado, Denver). The DBA/2J inbred mouse strain develops iris pigment dispersion, iris atrophy, and a spontaneous optic neuropathy that has many features in common with human glaucoma. All mice were between 4 months and 21 months of age. After euthanization, eyes were enucleated and held for imaging either in phosphate-buffered saline (PBS; 8 g/L sodium chloride, 0.2 g/L potassium phosphate monobasic, 2.16 g/L sodium phosphate dibasic heptahydrate, pH 7.4) for unfixed whole eye imaging or in 4% paraformaldehyde in PBS for fixed whole eye imaging.

**FITC-Dextran Perfusion**

In a single experiment, a eutnatanized C57BL/6 mouse (4 months of age) was immediately perfused from a gravity-fed catheter with a 2.5-ml solution of 20 mg/ml fluorescein isothiocyanate (FITC)-labeled dextran (average molecular weight, 150 kDa; Sigma-Aldrich, St. Louis, MO) in PBS. The dextran solution was perfused through a 26-gauge needle placed near the apex of the left ventricle of the heart until the liver of the mouse appeared greenish-yellow from the fluorescein dye under white lighting. The eyes were then immediately enucleated and preserved fresh in PBS on ice until imaged by 2PM (within 0–3 hours).

**Two-Photon Microscopy Imaging**

Except when noted, all enucleated mice eyes were oriented with the anterior/posterior axis perpendicular to the imaging axis, yielding sagittal/transverse cross-sections of the eye. 2PM imaging was performed using an adapted confocal microscope (LSM 510 META on Axiocam 200M platform; Carl Zeiss MicroImaging Inc., Göttlingen, Germany) with Zeiss 510 control software (Zen 2009; Carl Zeiss MicroImaging Inc.) equipped with a tunable mode-locked Ti:Sapphire laser (Chameleon Ultra II; Coherent Inc., Santa Clara, CA). The excitation source (Ti:Sapphire laser) was operated at a centered wavelength of 800 nm with 100- to 200-femtosecond pulses at a repetition rate of 80 MHz. 2PM was performed by focusing the excitation laser through the sclera of the mouse eye using a 25×/0.8 NA objective lens with a 0.21-mm working distance (LCI Plan-NeoFluar; Carl Zeiss MicroImaging Inc.). The emitted signal was first passed through a 650-nm short-pass filter to remove residual excitation of laser light. Unless otherwise noted in the text, 2PM signals were separately detected using non-descanned detectors with filter windows of 390 to 410 nm for SHG and 450 to 650 nm for 2PAF. Multiple x-y images were collected along the z-axis (image stacks) and processed in the control software (Zen 2009; Carl Zeiss MicroImaging Inc.). The laser power used in this study was between 24% and 28% of maximum laser power (>3.5 W), measured to be between 10 and 12 mW at the objective.

In the experiment performed on the FITC-dextran-perfused C57BL/6 mouse, the eye was first imaged by SHG and 2PAF, as described. Because FITC has a sharp fluorescence peak near 525 nm (Sigma) and melanin exhibits a broad fluorescence ranging from approximately 500 nm to approximately 700 nm, the two signals will overlap in the 2PAF channel (450 – 650 nm). To distinguish between these two signals, the FITC-dextran-perfused eye was also imaged using the attached spectral detector (META, Carl Zeiss MicroImaging Inc.) with user-defined channels of 516 to 548 nm for FITC-dextran fluorescence and 612 to 666 nm for melanin fluorescence.

**2PM Image Analysis and Processing**

The control software (Zen 2009; Carl Zeiss MicroImaging Inc.) package was used to prepare all 2PM images and animations. This software was used to integrate the two fluorescent signals into single dual-color images and to adjust overall image brightness (see Figs. 2A, 4B, 4C, 5A–D, 6A, 6B, 7A–C). Image stacks were projected into three dimensions using the Render series function to create 3D animations of the stacks seen rotating about the y-axis (Supplementary Movies S1–S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-7115/-/DCSupplemental). The Render series function was also used to create a virtual cross-section of the image stack (an x-z image), essentially by rotating the stacked images 90° about the y-axis (see Fig. 2B).

**Histologic Staining and Photography**

After 2PM imaging was completed, the eyes (fixed or fresh) were preserved in 4% paraformaldehyde in PBS overnight at 4°C. They were embedded in paraffin and sectioned at a thickness of 6 μm. Tissue sections were then stained with Mayer’s hematoxylin and eosin Y (H&E; Richard-Allan Scientific, Kalamazoo, MI). Bright-field imaging (see Figs. 1A, 1B, 4D) was performed using an advanced research microscope (Eclipse 80i; Nikon, Melville, NY) equipped with a color camera (D5-Fi1; Nikon) and a 10×/0.30 Plan Fluor objective lens (Nikon).

**RESULTS**

Histology sections clearly demonstrate the anatomy of the aqueous outflow region of the mouse eye. Figure 1 shows...
H&E-stained thin sections of the iridocorneal angle of a C57BL/6 mouse (Fig. 1A) and a DBA/2J mouse (Fig. 1B) with the following structures labeled: iris (IR), TM, and Schlemm’s canal (SC). The normal C57BL/6 mouse (Fig. 1A) contained a clear SC with clearly visible cellular TM (arrowhead). In contrast, the glaucomatous DBA/2J mouse (Fig. 1B) demonstrated marked corneal and scleral edema, hyperpigmentation of the iris (IR) with complete iridocorneal angle closure, and the presence of erythrocytes in the SC structure (arrowhead). Furthermore, the TM of the DBA/2J mouse could not be distinguished in the region of the iris insertion, and SC (arrowhead) appeared shrunken or collapsed. These changes were consistent with the known ocular pathology of this mouse strain, which includes pigment granule accumulation in the TM and eventual blockage of the drainage structures, leading to increased IOP and an eventual optic neuropathy that resembles human glaucoma.14

For subsequent imaging of freshly enucleated C57BL/6 eyes, the globes were oriented with the cornea perpendicular to the microscope objective, as shown in the schematic pictured in Figure 3. Automated tiling of the 2PM images allowed for a “bird’s eye view” of the entire cross-section of the C57BL/6 eye, enabling anatomic localization based on gross landmark identification (Fig. 4A). This technique was used to locate and scan the iridocorneal angle accurately and reproducibly (Figs. 4B, 4C). Figure 4A is a composite image of 5 × 5 tiles (1 tile = 450 μm × 450 μm) at a depth of 160 μm from the outer surface of the sclera. 2PAF (red) and SHG (blue) were simultaneously detected after excitation at 800 nm. The most abundant 2PAF signal came from iris pigment granules. SHG collagenous structures are visible from the cornea (top of the image), the sclera and choroid (sides and bottom), and conjunctiva and muscle (outside the globe). SHG collagenous structures are visible from the cornea (top of the image), the sclera and choroid (sides and bottom), and conjunctiva and muscle (outside the globe). Figure 4B is a single-frame 2PM scan of the right iridocorneal angle region corresponding to inset B in Figure 4A. The melanin from the peripheral iris (IR) was visible as 2PAF in the upper left of the panel (arrowhead), the cornea was visible as textured SHG at the top of the image, and the sclera was visible as dense SHG signal in the bottom right of the panel. SC was identifiable in the central area of panel B as a large region lacking both the SHG signal of the sclera and 2PAF from the iris (arrowhead). Adjacent tubeshaped regions within the sclera lacking SHG signal are consistent with the size and location of collector channels (CC, arrowheads). These CCs were visible radiating to the right toward the conjunctival surface, away from SC. From the region shown in Figure 4B, an image stack was collected at 1-μm intervals, encompassing a total depth of 40 μm, centered about the plane imaged in Figure 4B. These images were rendered in three dimensions, rotated about the y-axis, and the resultant animation is shown as Supplementary Movie S1 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-7115/-/DCSupplemental).

FIGURE 2. SHG in the mouse cornea. 2PM imaging was performed in an unfixed, enucleated C57BL/6 mouse eye imaged with the cornea toward the objective lens. (A) A single coronal cross-section showing the corneal stroma extracellular matrix imaged by SHG. The SHG signal exhibited an interlocking pattern that has been similarly reported in the literature. This 2PM image was captured at a depth of 90 μm from the anterior surface. (B) A virtual transverse cross-section built from a 90-μm deep image stack of 2PM images taken through the cornea. Scale bars, 100 μm.

FIGURE 3. Orientation of enucleated mouse eyes for imaging. Except where noted, all enucleated mouse eyes were imaged with the anterior/posterior axis of the eye perpendicular to the imaging axis. This yielded sagittal/transverse cross-sections of the eye (pictured).
Figure 4C is a single frame 2PM scan of the left iridocorneal angle region corresponding to inset C in Figure 4A. The melanin of peripheral iris (IR) was visible as the 2PAF signal of the melanin contained within these cells (arrowhead), the corneal stroma was visible as the SHG signal at the top of the panel, and SC was identified as the area lacking SHG signal near the insertion point of the IR. The thin mouse trabecular meshwork (TM) layer was identifiable as a hazy strip of SHG signal interior to SC. (D) Hematoxylin and eosin–stained histologic section from the same region shown in C. The relative locations of the SC, TM, and IR in this histologic section are in close approximation to their locations in the 2PM image. Scale bars, 100 μm.
nificant attenuation of 2PAF or SHG when imaging fixed tissue compared with unfixed tissue (data not shown). Figure 6 demonstrates 2PM of an intact fixed DBA/2 J mouse eye orientated approximately 30° to the surface of the limbus, with the purpose of imaging along the axis of the mouse iris. Figure 6 shows two adjacent image planes, separated by 3 μm. The image in Figure 6A is located interior to the image in Figure 6B. Panel A is at or near the surface of the TM because both the SHG signal from the interior corneal/scleral surface and the 2PAF signal from the melanin are visible. Located just exterior to this in panel B is a visible open pore consistent with the location of the junction of the SC with a CC (arrowhead). The large amount of overlying melanin (seen with 2PAF) and the difficulty in locating open structures within the sclera suggest that many of these pores are obstructed or collapsed. Furthermore, no large SC structures could be visualized in the iridocorneal angle of the DBA/2 J mouse. We believe our 2PM images, showing the obstruction or loss of fluid-filled structures with the sclera, are highly consistent with the histology shown in Figure 1B.

To verify that the regions lacking SHG signal in our 2PM images were aqueous-humor filled spaces, we perfused a C57BL/6 mouse with FITC-labeled dextran immediately after euthanatization and before enucleation of the eyes for imaging. Dextran-labeled blood vessels were present in the sclera near the corneoscleral junction. Figure 7A reveals several of these dextran-labeled vessels as the FITC conjugate was detected in the 2PAF detector range (450–650 nm; green). One major branch terminated near a patch of autofluorescent tissue in the SC wall. This is best visualized in the animation sequence of Supplementary Movie S3 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-7115/-/DCSupplemental). Supplementary Movie S3 is an animation of a 3D rendering of a 64-μm-thick image stack collected at 1-μm intervals through the left angle drainage region. The center image of this stack was the source of the image in Figure 7A. As the animation rotated, numerous signal-absent structures were in the sclera corresponding to channels that did not label with the dextran. These open structures did eventually connect with dextran-containing vessels similar to the pathway of collector channels and aqueous veins that connected with episcleral vessels in humans. Given that FITC fluoresces in the range used to detect the 2PAF signal from melanin, both are depicted as green in Figures 7A and S3.

Figure 7B is a subset image of Figure 7A, showing how the melanin and FITC-dextran fluorescent signals overlap. 2PAF signals are visible both from a tubular structure, likely a blood vessel located in the scleral wall, and from melanin located
Inadequate drainage of aqueous humor is believed to lead to elevated IOP, a leading risk factor for the development of glaucoma. In the conventional outflow pathway, the fluid seeps through the TM, flows through SC, and then flows into CCs. OCT and UBM have resolution within the anterior segment of approximately 15 to 25 μm. These imaging techniques lack the high resolution to accurately image the fine structures of the TM and C. We propose that 2PM is a uniquely qualified technique for the high-resolution acquisition of images necessary for monitoring the progression of primary open-angle glaucoma (POAG) by imaging the aqueous outflow system.

The complex morphology of the connective tissue and cells in the region of the TM and C may have significant consequences for the normal outflow of aqueous humor as well as the pathologic blockage of its drainage. Extracellular plaques deposited within the juxtacanalicular TM and the inner wall of SC were first described in electron microscopic analysis of glaucomatous eyes. The presence of these sheath-derived (SD) plaques was shown to be dramatically increased in trabeculectomy specimens from POAG eyes compared to normal eye specimens, with no correlation to age. Previous experiments pinpoint the juxtacanalicular TM region as a major site in outflow resistance. In normal-tension glaucoma, pathologic morphology in the TM region has also been demonstrated, including lattice collagen cluster deposits and a large amount of deposited SD plaque beneath the endothelium of SC. These findings and others underscore the importance of obtaining high-resolution structural images to monitor changes occurring in the TM that relate to the development of many types of glaucoma. The ability of 2PM to image collagenous tissue by SHG and autofluorescent structures by 2PAF demonstrate that it may be well suited to detect the changes noted here that occur in the glaucomatous eye.

In this study, we demonstrated the use of 2PM for imaging the iridocorneal angle tissue morphology of intact enucleated mouse eyes. Because of their different spectra, we were able to separate the SHG signal resulting from collagen structures from the 2PAF signal resulting from both the tissue melanin and the exogenously added FITC-dextran. With the numerical aperture of our objective lens and an excitation wavelength of 800 nm, we estimate that our imaging had an estimated lateral resolution of approximately 0.6 μm, with a depth resolution of approximately 3 μm. Thus, we were able to image intact mouse tissue with a resolution near that of histologic sections but without the distortions caused by an infusion of fixatives, tissue shrinkage from alcohols, and fine tissue morphologic changes that can occur with the infusion of paraffin or epoxy.

SC, C, and transscleral/episcleral vessels were clearly identified. Structural details of the collagenous corneal stroma match what has been reported for 2PM imaging in other species. The presence of TM and SC in the 2PM scans in this study demonstrates the usefulness of using mouse eye tissue as a suitable animal model for 2PM analysis of aqueous drainage pathology. Detection of significant 2PAF at the iridocorneal angle in the pigment-overproducing DBA/2J mouse in this study suggests this mouse strain as a useful model for

**DISCUSSION**

The current report demonstrates the usefulness of 2PM for transscleral imaging of the intact mouse eye and for identifying structures in the region of the TM. These findings represent, to our knowledge, the first high-resolution image acquisition of mouse aqueous outflow structures in unfixed tissue, and they provide the framework for advanced aqueous drainage studies in the mouse eye in vivo. These findings build on our previous work showing high-resolution deep tissue 2PM imaging of the human TM region.

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tracking glaucomatous changes by 2PM. Fluorescent dextran allowed for the visualization of some, but not all, vessels in the sclera near the limbus, leaving SC and CCs unlabeled. A more rigorous categorization of these channels with multiple labeling compounds may enable detailed characterization of outflow pathways by 2PM.

The potential to image the eyes of normal and DBA/2J mice, without sacrificing them for histology, could add to our understanding of aqueous outflow and would demonstrate the usefulness of 2PM for diagnosing glaucoma or characterizing its progression.

Acknowledgments

The authors thank Mark Petrash and Moshe Levi for providing the mouse eyes obtained for this study.

References

17. van der Merwe EL, Kidson SH. Advances in imaging the blood and aqueous vessels of the ocular limb. Exp Eye Res. 2010;91:118–126.


