In Situ Autofluorescence Visualization of Human Trabecular Meshwork Structure

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PURPOSE. To characterize the three-dimensional (3-D) structure of the human trabecular meshwork (TM) by two-photon excited (TPEF) autofluorescence (AF) and optical sectioning without conventional histologic embedding and sectioning.

METHODS. Viable human ex vivo explants of the anterior chamber angle containing the aqueous humor drainage tissue in situ were imaged by TPEF to localize AF and Hoechst 33342 nuclear fluorescence. An autofluorescent marker in Schlemm's Canal (SC) aided SC situ visualization. En face and orthogonal views of the TM were generated.

RESULTS. In the innermost uveal TM, AF signals outlined an intricate 3-D network of fine branching beams with large openings between the beams. In the adjacent corneoscleral TM, beams were thicker and coalesced as plate-like structures with pore-like openings. Linear and coiled AF fibers were visible on the background AF of beams. Deeper, in the external TM, this organization changed to fine fiber arrays orientated in the tissue's longitudinal axis, reminiscent of the cribriform plexus of the juxtacanalicular TM (JCT). In the outermost JCT, AF of fine fibers was sparse, then undetectable as optical sections approached the inner wall of SC. Cell nuclei were closely associated with the TM structural extracellular matrix.

CONCLUSIONS. We have used TPEF and optical sectioning to exploit AF as a useful method to visualize the structure of the human conventional aqueous drainage pathway in situ. Ancillary nuclear staining allowed cell association with the AF structures to be seen. This approach revealed a unique 3-D perspective of the TM that is consistent with known TM structural characteristics. (*Invest Ophthalmol Vis Sci.* 2012;53:2080-2088) DOI:10.1167/iovs.11-8141

The trabecular meshwork (TM) has a complex three-dimensional (3-D) design that is hard to capture in situ or replicate in vitro. This 3-D design supports the cells, structure,

and function of the tissue in regulating aqueous humor drainage.¹⁻⁵ Biological studies would be more authentic if the 3-D environment of the TM could be retained in studies.

A signature feature of the TM is its intricate internal 3-D network of branching beams that gives the tissue a mesh-like appearance microscopically.^{3,6,7} Arrangement of the 3-D extracellular matrix (ECM) network within the TM varies by depth, reflecting the tissue's anatomical transition from its uveal to corneoscleral to juxtacanalicular (JCT) parts (Fig. 1). The aqueous humor drains from the anterior chamber through these parts before entering Schlemm's canal (SC).^{1,3}

In the uveal and corneoscleral meshwork, branching beams have a core of structural ECM consisting largely of collagen^{8,9} and elastic fibers.⁹⁻¹¹ In the JCT, instead of a network of branching beams, arrays of fine elastin-like fibers create what is called the cribriform network.^{2,5,7} The JCT in the posterior TM overlies SC where it contributes resistance to aqueous humor drainage,³ while the anterior TM overlies sclera in the absence of SC.

A cell's 3-D environment is an important determinant of its behavior,^{12,13} but conventionally, TM cell biology studies have been conducted in two-dimensional cell culture partly because the complex 3-D architecture of the TM is difficult to replicate in vitro. A possible solution to this difficulty is to develop biological insights in the intact 3-D architecture of the drainage tissue in situ. We have begun to explore this possibility using two-photon excitation fluorescence (TPEF) microscopy,^{14,15} a technique that may be useful for this purpose.¹⁶⁻¹⁹

TPEF was used to characterize autofluorescence (AF) signals within the TM of ex vivo human post-corneal transplantation tissue. Elastic fibers and certain collagens of the structural ECM of the TM are endogenous fluorophores that are autofluorescent by TPEF in the absence of exogenous labeling.¹⁴ We have studied the attributes of AF in the in situ TM to determine what these endogenous signals reveal of the tissue's fine structure.

MATERIALS AND METHODS

Human Donor Tissue

Human donor corneoscleral rim tissue was generously provided by Doheny corneal surgeons. Corneal transplantation typically occurred within 6 days postmortem (personal communication, Dr. Martin Heur, August 19, 2011). Procurement was approved by the Institutional Review Board of the University of Southern California and complied with the Declaration of Helsinki. For institutional regulatory reasons, no accompanying information on the donor tissue was available to us apart from the date of corneal transplantation surgery. Donor tissue was received immediately after corneal transplant surgery, maintained in Optisol GS transport media (Bausch & Lomb, Rochester, NY) at 4°C, and processed right after receipt. A central button of cornea had been removed, leaving the TM and SC outflow system intact within the donor tissue.

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FIGURE 1. Cutaway diagram of the TM. *Right to left:* uveal, corneoscleral, and juxtacanalicular meshwork and SC. *Bottom left:* SC borders the posterior TM, next to the iris root and scleral spur. *Top right:* the anterior TM borders the peripheral cornea. Adapted with permission from Allingham RR, Damji KF, Freedman S, Moroi SE, Shafranov G, eds. Figure 1.10. In: *Shields' Textbook of Glaucoma*. 5th ed. Wolters Kluwer: Philadelphia, PA 19103: Lippincott Williams & Wilkins. 2005: 20.

Tissue Preparation

Tissue was placed with the TM side up in Optisol GS on a glass dish and segmented into 8 to 12 wedges of 2 to 4 mm width. When donor blood reflux was present in SC, it provided a convenient marker for locating the TM (Fig. 2, asterisks). In some specimens, SC was cannulated with a 6-0 nylon suture (Ethilon black monofilament, Ethicon, Somerville, NJ) that was gently inserted into the lumen aided by a dissecting microscope (see Supplementary Material and Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8141/-/DCSupplemental>). The suture emitted AF between 500 and 550 nm with TPEF excitation at 850 nm (see Fig. 3).

We evaluated postmortem tissue for optimal quality and cellularity by the following criteria: (1) Tissue that was fresh and firm, but not rigid (possible protein cross-linking) or flaccid (architectural breakdown) was analyzed further. We have found that the latter correlates with altered TM structures and reduced cellularity. (2) Tissue with abnormal trabecular beam-associated autofluorescent aggregates during TPEF imaging was excluded. This type of tissue usually had reduced cellularity and was (3) with disordered and diminished TM beam autofluorescence (AF) seen. (4) TM cellularity was assessed by Hoechst nuclear DNA staining. Based on observation in recent postmortem tissue, cell density in the corneoscleral meshwork of at least 70 to 100 cells per $246 \times 246\text{-}\mu\text{m}$ image frame was considered acceptable. Tissue with lower cellularity or evidence of nuclear fragmentation was excluded. (5) If cell viability was questionable, further vital dye evaluation was conducted with Calcein AM, a cytosolic vital dye, and counterstaining with propidium iodide for necrotic and apoptotic nuclei.^{20,21} (6) The TM of fresh whole eyes were imaged within 48 hours postmortem as a reference standard against which to compare TM images from the postmortem transplantation tissue we received. Tissue was considered suboptimal due to factors such as inadequate postmortem tissue preservation, perisurgical handling, and postmortem age.

FIGURE 2. Light microscopy of ex vivo human donor corneoscleral tissue wedge showing angle structures marked as (A) region of ciliary muscle attachment, (B) TM overlying SC (here, blood [red; *] has refluxed into SC making it easy to identify); and (C) cornea. Bar = 1 mm.

TPEF Setup

A Leica TCS SP5 AOBS MP confocal microscope system (Leica Microsystems, Heidelberg, Germany) coupled to a Chameleon Ultra-II multiphoton laser (Coherent, Santa Clara, CA) was used. Tissue wedges were placed TM-side down onto a glass-bottom microwell dish. Incident light was focused and emitted signals were collected with inverted HCX PL APO CS 63X/1.3 NA glycerol or 20X/0.7 NA long-working distance objectives (Leica). Excitation of AF and Hoechst 33342 fluorescence was at 850 nm. TPEF signals were collected in epifluorescence configuration, split with dichroic mirrors, and guided through multiphoton bandpass filters (TPEF = 525/50 or 500–550 nm; epifluorescence = 585/75 or 550–625nm; Leica) and a non-descanned photomultiplier tube (PMT) detector (NDD, Hamamatsu, Bridgewater, NJ). Images were collected as Z-stacks (xyz, 600 Hz, bidirectional) using 512×512 or 1024×1024 pixel resolution and $16\times$ line averaging. Virtually the whole TM was imaged in 8 to 12 separate segments (30° – 45° each) around the tissue's circumference. One to two Z-stacks were captured per segment. Wide-field AF was captured by Argon laser excitation at 488 nm and emission detection at 500 to 535 nm using an internal PMT detector. Images were analyzed with Velocity 5.4.1 (PerkinElmer, Waltham, MA) or LAS AF Lite 2.2.1 (Leica). Images were cropped, resized, and fit into figures using Photoshop CS5 (Adobe, San Jose, CA).

We report representative observations made in over 50 separate donor eyes. Supplemental Figure S2 shows TM AF images of fresh reference eyes (imaged within 48 hours postmortem; $n = 2$) and representative 6-day-old transplantation tissue. The donor tissue we used looked similar to reference TM AF images. AF images from four separate quadrants of the uveal, corneoscleral, and juxtacanalicular TM were also similar within and between eyes. The fresh reference eyes and 6-day-old transplantation tissue were stained with Hoechst 33342 nuclear label and found to have similar cellularity (data not shown). Supplemental Figure S3 compares AF images from viable and nonviable TM. In poor quality nonviable TM, AF beams with altered configuration, disrupted fibers, and abnormal autofluorescent aggregates were seen.

FIGURE 3. AF in an optical section through a 6-0 nylon suture outside ocular tissue. Emission was captured through 585/75 (left: 550–625 nm, red) and 525/50 filters (right: 500–550 nm, green). TPEF excitation at 850 nm; original magnification $\times 630$; bar = 25 μm .

RESULTS

Figure 2 shows *ex vivo* human ocular tissue with the angle structures *in situ*. The region of ciliary muscle attachment (A) bordered the TM (B) overlying SC (red; refluxed blood in SC). The TM (B) was between ciliary muscle (A) and peripheral cornea (C). This region (A–C) was imaged by TPEF.

Figure 3 shows nylon suture AF outside the tissue. AF was visible with a 525/50 (right; green channel) but not 585/75 bandpass filter (left; red channel).

Figure 4 shows low power ($\times 20$; 500–550 nm bandpass filter (blue-green) wide-field AF microscopic images of the TM. In Figure 4A, the TM lay deep to the dark gap seen between the cornea (below) and ciliary muscle (above). The cornea (below in image) was located anterior to the TM in the anterior chamber angle and had relatively low AF (see later Fig. 4C) apart from just adjacent to the TM, a region consistent with the anatomical Schwalbe's line.¹⁹ The ciliary muscle (above in image) was located posterior to the TM in the angle. The beginnings of the branching beams of the uveal meshwork were visible adjacent to the ciliary muscle. The rest of the TM was draped like a hammock below this plane as depicted in the orthogonal image, where the inner TM surface was slightly external (deep) to the plane of the ciliary muscle and corneal surface. In Figure 4B, 25 μm external to image Figure 4A, an interconnected network of fine branching TM beams traversed the space between the cornea and ciliary body. Spaces between branching beams were large. This was the uveal meshwork. In Figure 4C, 20 μm external to the image of Figure 4B, beams were thicker and coalesced like sheets with pore-like openings that were smaller than the inter-beam spaces in Figure 4B. This TM region was consistent with the corneoscleral meshwork.

Figure 5 shows higher magnification fluorescence microscopic images of the angle structures of Figure 4. Figure 5A shows a region where wavy autofluorescent fibers from the ciliary muscle (asterisk; above in image) interfaced with autofluorescent fibers of interconnected TM beams (double asterisk; below in image). The fibers from ciliary muscle were orientated perpendicular to the longitudinal orientation of the TM and SC. Figure 5B shows interconnected autofluorescent beams and fibers of the inner TM. Figure 5C shows the region of cornea adjacent to TM. Hoechst 33342-stained nuclei were arranged as a regular mosaic at the inner corneal surface.

Spaces between nuclei were dark, indicating that the cornea here had low background AF.

Figure 6 shows AF optical sections through the TM from its inner to outer region to SC, as excited with 850-nm (Figs. 6A–F) and 750-nm (Figs. 6G–J) wavelengths. Figures 6C–F and 6G–J are matching image frames, all captured in rapid succession. A structural transition from uveal (Fig. 6A) to corneoscleral (Fig. 6B) to juxtacanalicular (Fig. 6C) meshwork was seen. Figures 6D–E are image sections through the JCT/SC inner wall endothelium interface showing cell nuclei (matching Fig. 6G–J). Evidence of nuclear staining was lost just 1 μm external to Figure 6E (matching Fig. 6I). These data are not shown but appear similar to the optical section of Figure 6F (matching Fig. 6J) that was 20 μm deeper than Figure 6E (matching Fig. 6I) and lay wholly in SC. Compared with Figures 6C–E, Hoechst 33342-stained nuclear fluorescence in Figures 6G–J was more intense, but TM fiber and SC nylon AF was less intense, such that cell nuclei in the JCT/SC region were more easily seen using 750-nm excitation. The inner wall of SC was identified by a final layer of nuclear fluorescence approximately 80 μm from the inner surface of the TM. More external to this, in the SC lumen, no nuclear fluorescence was seen.

Figure 7 shows AF differences between the posterior TM (left column, A–E; next to ciliary muscle and scleral spur; overlying SC) and anterior TM (right column, F–J; next to cornea; overlying sclera but not SC). The inner layers (uveal [Figs. 7A and F] and corneoscleral meshwork [Figs. 7B–D and 7G–I]) of the anterior and posterior TM look similar, but the outer layers of the anterior (Figs. 7H–J) and posterior TM (Fig. 7E) look different. In the posterior TM, the outer region had fine fiber arrays aligned with SC resembling a cribriform network consistent with the JCT.⁷ In the anterior TM, the corneoscleral meshwork transitions to a region crisscrossed with randomly aligned fine autofluorescent fibers appearing on a more homogenous background having an organization resembling sclera²² (Figs. 7I–J). Figure 8 shows details of the thin branching beams in the uveal meshwork of the inner TM. Spaces between branching beams often exceeded 100 μm in diameter. The thickness of the uveal meshwork *in situ* was estimated at $40.6 \pm 10.0 \mu\text{m}$ ($n = 5$). In Figure 8A, the branching beams traversed the distance between cornea and ciliary muscle. In Figure 8B, the branching beams are seen at higher magnification. External to and interspersed amongst the branching autofluorescent trabecular beams were finer, more

FIGURE 4. Single-photon confocal microscopy showing AF in the angle structures. TM lies between the ciliary muscle (*above*) and cornea (*below*). (A) AF from the ciliary muscle and cornea. Adjacent orthogonal image shows TM lay deep (more external) to structures in the optical section. (B) Slender branching TM beams with large gaps between beams at 25- μ m depth. (C) Coarser autofluorescent beams with smaller intervening gaps at 45- μ m depth. Original magnification $\times 200$; bar = 100 μ m.

linear nonbranching autofluorescent fibers (arrows). Figures 8C–I are magnified images of the beams showing details of the autofluorescent signal in these structures. The AF is heterogeneous. Figures 8C and 8I show fine autofluorescent fibers orientated within beams and aligned with their axes, surrounded by less intense AF within each beam. In Figures 8C–H, further AF signals are evident as fine protuberances along the edge of the autofluorescent beams. These protuberances recur

FIGURE 5. Two-photon excitation fluorescence of adjacent tissues in the angle. (A) Wavy autofluorescent fibers from ciliary muscle (*) adjacent to branching TM beams (**). (B) Autofluorescent branching beams in the TM. (C) Hoechst 33342-stained nuclei of the corneal endothelium. TPEF excitation at 850 nm; original magnification $\times 630$; bar = 25 μ m.

FIGURE 6. AF feature transition through the same tissue region. Two-photon excited AF is by 850 nm (A–F) and 750 nm (G–J) laser excitation. (A) slender beams and large inter-beam gaps in the uveal meshwork (TM; cross-hair, orthogonal reconstruction). (B) Deeper (more externally), in the corneoscleral meshwork, AF beams are thicker, giving a plate-like appearance with pores. (C–D) In the JCT, arrays of autofluorescent fibers without pores are orientated along the longitudinal axis of the TM. Hoechst 33342-stained nuclear fluorescence is seen. (E) Region of the inner wall endothelium of SC. Nylon suture AF in the SC lumen is just visible. AF fibers in the adjacent JCT are parallel to SC. (F) Nylon AF within the SC lumen. (G–J) Hoechst 33342-stained nuclear fluorescence intensity is greater by 750 nm than 850 nm excitation (C–F; matching images). AF intensity of the nylon suture and TM beams and fibers (G–J; dashed outline in SC) is less compared with 850 nm excitation (C–F). (I) Nuclei and nylon suture AF in the SC inner wall region are seen together (compare with Fig. 6E). (J) Deeper, within SC, nylon AF is seen but not nuclear fluorescence (compare with Fig. 6F). Original magnification $\times 630$; bar = 25 μm .

FIGURE 7. AF features of the posterior (A–E) and anterior (F–J) TM. (A) Uveal, (B–D) corneoscleral, and (E) JCT regions of the posterior TM. (F) Uveal and (G–H) corneoscleral regions of the anterior TM adjacent to (H–J) sclera. (H–J) The corneoscleral meshwork abruptly transitions to tissue having more homogenous AF, crisscrossed by randomly aligned fine fibers. Original magnification = $\times 630$; bar = 25 μm .

FIGURE 8. AF detail of the uveal meshwork. (A) Wide-field AF optical section ($\times 200$). TM lies between ciliary muscle (above; posterior) and cornea (below; anterior). (B) Branching beams and separate fine fibers (*arrows*) in the uveal meshwork ($\times 630$). (C–I) Heterogeneous AF signals in beams. Beam edges had autofluorescent protuberances with a periodicity of 5 μm (C, E–G; *arrowheads*) that were part of coil-like formations associated with beams (D, E [*arrowheads*], H). Bright autofluorescent linear fibers were present in beams (C, E–G, I). TPEF excitation at 850 nm.

at regular intervals with a periodicity of about 5 μm and are part of coil-like signals on the beams (*arrowheads*).

Figure 9 shows details of thicker trabecular beams in the corneoscleral meshwork. The slender beams of Figure 8 have transitioned to thicker beams with smaller inter-beam spaces (Figs. 9A, 9B; left: wide-field; right: higher magnification). More externally, the thicker beams appeared coalesced in a plate-like arrangement with even smaller openings resembling pores (Figs. 9C–I). As in Figure 8, fine autofluorescent fibers were present within the beams and plate-like structure and surrounding pores. Figures 9D–I are serial optical sections through 5 μm of a corneoscleral plate showing the course of several pores. A pore is highlighted (single cross) to show its uninterrupted course through the tissue; a separate pore (double cross) appeared to end blindly or possibly change its course in the tissue.

Figure 10 shows the outer TM where autofluorescent fiber arrays were aligned with the longitudinal axis of the TM and SC (Figs. 10A, 10B). Hoechst 33342 nuclear staining depicted cellularity. In Figure 10B at higher magnification, arrays of fibers resembling the cribriform network^{1,6} were seen in the JCT. Figures 10C and 10D, at higher magnification, show that autofluorescent fibers originating from the ciliary muscle were orientated perpendicular (running vertically) and intermingled (circles) with the autofluorescent fiber arrays (running horizontally) of the JCT/outer-TM region. The tissue thickness of this TM region was estimated at $14.8 \pm 6.2 \mu\text{m}$ ($n = 5$).

FIGURE 9. AF detail of the corneoscleral TM. (A) Wide-field AF ($\times 200$). Nuclei are stained with Hoechst 33342. (B) Beams and fibers of the corneoscleral TM ($\times 630$). (C) Plate-like appearance with pores in deeper location. Bright, fine fibers were resolved within the plate-like structure. (D–I) One-micrometer-step serial sections through region indicated by asterisk in Figure 9C. (D) Individual pores indicated by † and ‡. (E–I) One pore (†) was continuous through 5 μm . The other pore (‡) closed or diverted within 5 μm . TPEF excitation at 850 nm.

FIGURE 10. AF detail of the JCT in the outer TM. (A) Wide-field AF optical section ($\times 200$). Nuclei were stained with Hoechst 33342. (B) Autofluorescent fiber arrays in the outer TM ($\times 630$). (C) Fibers from ciliary muscle next to fibers of the JCT (within circles). (D) Orthogonal reconstruction showing depth location of Figure 10C of 78 μm (cross-hair location). TPEF excitation at 850 nm.

DISCUSSION

We have used TPEF and optical sectioning to visualize the human conventional aqueous humor drainage tissue *in situ*. TM endogenous fluorescence was exploited as an intrinsic signal for visualizing fine structural characteristics. This was achieved without conventional fixing, embedding, and physical sectioning. En face views of TM AF were easily obtained and could be used to reconstruct the tissue in different dimensions.

Imaging revealed an intricate 3-D network of interconnected autofluorescent trabecular beams and fibers whose arrangement varied as a continuum from the inner to outer TM. Exogenous labeling was not needed as the AF signal alone provided detailed structural views. The TM was distinguishable from the neighboring ciliary muscle and cornea.⁶ Placing an autofluorescent nylon marker within SC was a novel step that provided a critical landmark for the drainage canal. This permitted the JCT/SC interface to be identified *in situ*. A shortcoming of 6-0 nylon suture cannulation is that it caused some inward bulging of SC, with deformation of the adjacent TM, as seen in Fig. 6. If such deformation is to be avoided, the canal can be cannulated for only a short distance. The position of the downstream SC beyond the suture tip is then easily deduced. SC cross-sectional morphology beyond the suture tip is flatter, and the adjacent TM is undistorted. Visualizing the JCT and SC inner wall endothelium side by side could provide meaningful possibilities for studying biological interactions within this high resistance region.

The structural characteristics of the TM evident by AF mirrored prior descriptions by conventional light and electron microscopy and immunohistochemistry.^{1,2,4,5,7,23,24} In our case we used TPEF in fresh and unfixed tissue. In the innermost TM adjacent to the anterior chamber, AF outlined a 3-D network of fine branching beams reminiscent of the uveal meshwork.^{1,2,3,6} Adjacent and external to this (toward JCT/SC), autofluorescent beams were coarser. They then coalesced to form sheets with pore-like openings consistent with the corneoscleral meshwork. Though lacking AF, these pores are not necessarily empty but likely have associations with at least cells and proteoglycans.²⁵ The pore-like openings became smaller toward the JCT. Autofluorescent fibers were seen within beams and as coil-like shapes on beams. Separate fine autofluorescent fibers also ran external and alongside beams. The source of this heterogeneity is unclear but may reflect the conformation of elastic fibers, which are known to associate with TM beams and beam cores.⁹⁻¹¹ More externally in the TM, beams gave way to arrays of fine autofluorescent fibers, as has been described of the cribriform fiber network of the JCT.^{1,7} Throughout, cells were associated with the autofluorescent network of trabecular beams and fibers. Beyond the TM, regions such as the sclera (Fig. 7) and limbus (Fig. 4) also had AF, although it was more dense and homogenous than that of the TM. Factors such as different ocular tissue depth, tissue density, 3-D organization, and makeup of endogenously fluorescent structures need to be considered when optimizing imaging settings.

Elastin and the structural collagens are candidate sources of AF within the TM. The cores of trabecular beams contain structural proteins such as type I and type III collagen and elastin or elastin-like fibers.⁸⁻¹¹ These structural ECM proteins are natural fluorophores.¹⁴ It is thus possible that the AF we observed originated in the cores of trabecular beams. If so, it is likely that a more peripheral, subcellular region surrounding beam cores was not seen in our AF optical sections. This subcellular region may not contain autofluorescent structural ECM proteins such as type I collagen and would need exogenous labeling to be seen by TPEF. It would be

worthwhile to determine the specific fluorophores responsible for TM AF. This will be the focus of future studies. If the origin and localization of AF signals in the TM were known, the signals could be exploited to identify specific entities within the tissue.

The tissue AF signals ought to be distinguished from second harmonic generation (SHG), a nonlinear phenomenon occurring in two-photon microscopy.^{14,15,26} SHG is due to scattering and nonlinear optical recombination of photons instead of excited fluorescence emission. We used filters to detect AF emission at 500 to 550 nm. This is beyond the filter bandpass needed to detect the SHG of TM ECM proteins such as collagen (425 nm) and elastin (380 nm)¹⁴ for the respective two-photon stimulating wavelengths of 850 nm and 760 nm that we used. SHG for a particular protein is detectable only over a very narrow filter bandwidth for a given incident wavelength, and would not have been visible by the filters that we used.

The recently *ex vivo* corneoscleral tissue we imaged was of sufficient quality for human therapeutic transplantation. Based on prior studies we know that the postmortem TM in *ex vivo* organ culture is structurally and functionally viable in culture medium for at least 4 weeks;²⁷ organ-cultured postmortem eyes are successfully used for week-long aqueous drainage physiological studies;²⁸ transplant eye banks consider donor corneoscleral tissue viable for transplantation for up to 2 weeks postmortem as a standard of patient care (oral communication, California Transplant Services, Carlsbad, CA); and human TM cells can be explanted from donor corneoscleral rims and successfully established in primary culture over long periods postmortem.²⁹ Conversely, eye bank donor tissue was not perfused and may have been compromised during processing and storage. For institutional regulatory reasons, we did not have access to specific information on donor and postmortem age for the tissue we received and so cannot report this. Instead we empirically assessed tissue quality by manipulation, imaging representative samples for autofluorescent and structural clues of tissue compromise, DNA labeling, intravital dye cellular viability analysis,^{20,21} and using a reference set of images for comparison (see Materials and Methods). Tissue that did not meet our empirical standards was excluded from further analysis.

The *ex vivo* tissue was not imaged while under perfusion or pressure, and it is possible that the images do not exactly replicate TM morphology under physiological conditions. Under pressure, TM stretching, balanced by counteracting tensile forces from structural ECM proteins such as type I collagen, can be expected. This could alter the appearance of tissue pores, beams, and plate-like structures. Still, our *in situ* environment was not dissimilar to conventional histology, immunohistochemistry, and *in vitro* studies of TM cells, from which many valuable insights have been gained; all are often conducted under conditions not exactly mimicking the eye's physiological environment. TPEF, however, can be performed without the harsher processing and physical sectioning needed for conventional histologic processing. Conventional histologic fixation can also introduce background fluorescence that potentially confounds the relatively weak AF. TPEF allows for submicron optical sectioning although its resolution is still below that of electron microscopy. Photo damage is less, and tissue penetration is deeper compared with single-photon laser confocal microscopy. Hence, live cellular and deep tissue optical sectioning is possible without destroying the tissue. Three-dimensional reconstruction of structures can be performed. Recent reports suggest TPEF could be applied to the eye *in vivo*.¹⁶⁻¹⁸ We are proceeding to investigate if the viability of the *ex vivo* tissue is commensurate with conducting live dynamic cellular studies *in situ*.

Our use of postmortem transplant tissue simply and inexpensively salvages good quality and scarce human tissue for research in a way that is sustainable. If a model experimental system using this tissue could be designed to closely mimic the cell, ECM, and 3-D organization of the conventional drainage pathway, such a system could be used to develop more complete insights into the tissue's systems biology. It would be a technical advance if biological interactions could be examined directly, live, and quantitatively within the TM, providing a platform for noninvasively exploring cellular, molecular, and pharmacologic dynamics over time in biological specimens. Our findings here indicate that AF signals could serve as useful markers in studies of the viable TM in situ. We are proceeding to further explore and characterize pertinent biological aspects of the drainage tissue by the techniques described herein.

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