Two-Photon Immunofluorescence Characterization of the Trabecular Meshwork In Situ

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PURPOSE. To develop an in situ model to study biological responses and glaucoma pathology in the human trabecular meshwork (TM). Characteristic TM cell- and glaucoma-associated markers were localized in situ in relation to the tissue's autofluorescent structural extracellular matrix (ECM) by two-photon excitation fluorescence optical sectioning (TPEF).

METHODS. Human donor corneoscleral (CS) tissue containing the intact aqueous drainage tract was incubated with dexamethasone (Dex) or TGF- β 1, and immunostained for epifluorescence (EF) microscopy with antibodies to myocilin and alpha smooth muscle (α -SMA). Separate specimens were labeled for Type-IV collagen and fibronectin. Nuclei were stained with Hoechst 33342. Multimodal TPEF was used to visualize EF, intravital dyes, and autofluorescence (AF) in situ. Three-dimensional (3D) localization of fluorescence within the TM was analyzed using reconstruction software.

RESULTS. Autofluorescent beams, perforated sheets, and fibers, consistent with the uveal (UV), CS, and juxtacanalicular (JCT) meshwork, respectively, were captured at different depths of the TM. Type-IV collagen EF distinctly outlined the AF beams in a location consistent with basement membrane. Fibronectin EF showed a diffuse reticular pattern throughout the TM. TGF- β 1-induced α -SMA expression, which was distributed perinuclearly in cells among autofluorescent structures. Dex-induced myocilin expression had both cytosolic and extracellular distributions.

CONCLUSIONS. The authors have localized markers that are characteristic of TM cells and relevant to glaucoma pathogenesis in situ using multimodal TPEF without conventional histological embedding and sectioning. Protein expression was inducible in situ and could be analyzed with respect to cells and the ECM within the 3D environment of the human TM. (*Invest Ophthalmol Vis Sci.* 2012;53:3395-3404) DOI: 10.1167/iovs.11-8570

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The trabecular meshwork (TM) has a complex three-dimensional (3D) organization that facilitates its roles in resisting aqueous humor drainage and regulating IOP. The 3D organization of the TM is supported by an elaborate architecture of extracellular matrix (ECM), and cells whose interactions play vital roles in tissue function. While TM fluid dynamics are typically measured in vivo or in ex vivo organ culture, the nature of underlying molecular interactions cannot be directly observed in these models, but have to separately be deduced in vitro.

We are developing a tissue model in which to probe cell and molecular mechanisms in situ. We have sought to characterize a number of key proteins for identifying TM cells that are also relevant to glaucoma pathogenesis in this system. Fibronectin (FN) and type-IV collagen are ubiquitously expressed TM basement membrane proteins.¹⁻³ FN is increased in the TM of human eyes with POAG.⁴ The heparin 2 domain of FN increases outflow facility in ex vivo organ culture.^{5,6} Type-IV collagen accumulates in the TM of eyes with steroid-induced glaucoma.^{7,8} In cultured TM cells, the heparin 2 domain cooperates with type-IV collagen to alter actomyosin dynamics.⁹

Myocilin is another marker linked to glaucoma. Myocilin is upregulated by cultured TM cells exposed to glucocorticoids,¹⁰ and its induction by Dex is critical to characterizing cultured TM cells.¹¹ Mutations in the myocilin gene are linked to juvenile-onset open-angle glaucoma and a subset of adult-onset POAG.¹² Increased myocilin expression with glucocorticoid induction is found intracellularly and extracellularly, raising the possibility of functional or pathogenic roles for myocilin in both environments.¹³

Alpha-smooth muscle actin (α -SMA) is expressed in posterior regions of the TM and upregulated by TGF- β .¹⁴ Increased levels of TGF- β 1 and - β 2 in aqueous humor are associated with POAG.¹⁵ It is hypothesized that increased aqueous humor TGF- β and TM α -SMA expression increase outflow tissue contractility, which may contribute to elevated IOP¹⁶ and reduced outflow facility.¹⁷

We have applied two-photon excitation fluorescence optical sectioning (TPEF) to analyze cells, ECM, and biological responses in the intact human TM. They have modified in vitro labeling methods for in situ fluorescence whole tissue analysis. TPEF allows for sufficiently deep tissue imaging without resorting to traditional histological embedding and sectioning. Recently postmortem human donor corneoscleral (CS) tissue retained from corneal transplantation containing the intact conventional aqueous humor drainage tract was used. The goal for the proposed model system is to make it possible to observe cell-ECM interactions within their original 3D environment with the relatively simple bench-top accessibility afforded by in vitro approaches. To validate the in situ findings, we have sought to establish the tissue presence of inducible and noninducible markers that are characteristic of TM cells and pertinent to glaucoma cellular pathology.

MATERIALS AND METHODS

Reagents

Low glucose Dulbecco's modified Eagle's medium (DMEM), L-glutamine, Gentamicin, Amphotericin B, dimethyl sulfoxide (DMSO) and HEPES were purchased from Mediatech (Washington, DC). Penicillin/Streptomycin was purchased from the Norris Comprehensive Cancer Center Cell Culture Core (Los Angeles, CA). We used serum-free media comprising low glucose DMEM, streptomycin, penicillin, gentamicin and L-glutamine. Dex was purchased from EMD Chemicals (Gibbstown, NJ). Active TGF- β 1 was purchased from Abcam (Cambridge, MA).

Antibodies

Mouse-derived monoclonal anti-gial fibrillary acid protein (GFAP) clone GA5 (Millipore, Billerica, MA), mouse monoclonal anti-collagen 4 alpha 2 chain clone 23IIC3 (Millipore), rabbit polyclonal anti-FN (Sigma, St. Louis, MO), and rabbit polyclonal anti- α -SMA (Abcam) antibodies were used at a dilution of 1 to 100. GFAP is a class 3 intermediate filament and a cell-specific marker of astrocytes, and was used in this study as a negative control. Rabbit polyclonal antimyocilin (Abcam) was used at a 1 to 50 dilution.

Human Donor Tissue

Human donor CS rim tissue was generously provided by physicians of the Doheny/USC Corneal Service (26 unique donors). Procurement was approved by the institutional review board at the University of Southern California, and complied with the Declaration of Helsinki. Tissue was typically transplanted within 6 days postmortem (Martin Heur, oral communication, 2011). For institutional regulatory reasons, no accompanying information on the donor tissue was available to the authors apart from date of corneal transplantation surgery. Tissue was received immediately after corneal transplantation, during which a central button of cornea had been removed from the donor tissue. This left the entire TM and subcutaneous (SC) outflow system intact within the donor tissue. Tissue was processed for analysis immediately after receipt. The tissue was maintained and received in Optisol GS transport media (Bausch & Lomb, Rochester, NY) at 4°C.

Tissue Quality Screening

We evaluated postmortem tissue for optimal quality, cellularity, and viability, as this was important for the protein induction and expression studies. The following criteria were used: (1) tissue that was fresh and firm but not rigid (possible protein cross-linking) or flaccid (architectural breakdown) was used. It was found that the latter correlates with altered TM structures and reduced cellularity, (2) tissue with abnormal autofluorescent aggregates was excluded. This type of tissue usually had reduced cellularity, (3) disordered or diminished TM beam autofluorescence (AF), (4) TM cellularity was assessed by Hoechst intravital dye nuclear DNA staining. Cell density in the CS meshwork of at least 70 to 100 cells per 246- by 246- μ m image frame was considered acceptable. Tissue with cellularity less than this was excluded, and (5) If cell viability was questionable, further intravital dye evaluation was conducted with Calcein AM, a cytosolic vital dye for viability, and counter staining with propidium iodide (PI) for necrotic and apoptotic nuclei as shown in Figure 1 (22 donors).^{18,19} Tissue segments were incubated with 0.3 μ M Calcein AM and 1 μ g/mL PI in PBS (1 $\times$, pH 7.4) for 30 minutes at 37°C and 8% CO₂. Some wedges were killed with 0.2% Triton X-100 in PBS for 30 minutes prior to labeling to serve as dead controls. Tissue was considered suboptimal due to factors such as inadequate postmortem tissue preservation, perisurgical handling, and postmortem age.

FIGURE 1. Intravital dye live cellularity assays of donor human TM in the region of the CS meshwork. (A) Tissue killed with Triton X-100 showed prominent PI (red, nuclear) but no Calcein (green, cytosolic) labeling. Autofluorescent TM fibers are seen (green). (B) Viable tissue had a preponderance of Calcein labeling masking the dimmer fiber AF. PI labeling was scant. Bar equals 25 μ m.

Tissue Preparation, Fixing, and Staining

Tissue was placed with the TM side up onto a 150- by 20-mm glass dish (VWR International, Radnor, PA) along with 5 mL of Optisol GS to prevent drying. The tissue was cut into 8 to 12 segments 2 mm to 4 mm wide. For staining, segments were placed in 24-well plates, washed twice with 1 mL PBS, fixed for 30 minutes in 1 mL glutaraldehyde fixative (3.9% formalin, 0.5% glutaraldehyde, 0.2 M HEPES, pH 7.4), permeabilized for 2 hours in 1 mL of 5% Triton X-100 in PBS, rocking at 4°C, and blocked in 1% BSA for 30 minutes at room temperature. Wedges were placed in 96-well plates, and incubated with 150 μ L of primary antibodies in 0.1% BSA/PBS overnight (approximately 16 hours) at 4°C on a rocking platform. The wedges were transferred to 24-well plates, washed three times with 1 mL PBS, then transferred to sterile 96-well plates, and incubated with 150 μ L of Alexa 568-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Invitrogen, Grand Island, NY) in 0.1% BSA/PBS overnight at 4°C on a rocking platform. Wedges were washed twice with PBS and then incubated with Hoechst 33342 (Invitrogen) for 15 minutes at room temperature. Tissue from seven donors was used for fibronectin staining; 14 for type-IV collagen; 12 for myocilin; and 12 for α -SMA staining.

Tissue Treatments (Prefixation and Labeling)

(1) Dex: tissues (9 donors) were washed twice with 2 mL PBS and incubated with 2 mL serum-free media, and either 250 nM Dex or 1 to 2000 ethanol (vehicle control) for 3 days at 37°C and 8% CO₂. Dex, or vehicle, was added directly to the media daily and the media was changed every other day, and (2) TGF- β 1: tissues (7 donors) were washed twice with 2 mL PBS and incubated with 2 mL serum-free media and either 50 nM TGF- β 1 and 1% BSA or DMEM alone for 2 days at 37°C and 8% CO₂. The media was replaced on the second day.

Two-Photon Imaging Setup

Tissue wedges were imaged with 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). The wedges were placed TM side down, onto a glass bottom microwell dish. Incident light was focused, and emitted signals collected, with an inverted HCX PL APO CS 63X/1.3NA glycerol objective (Leica) or a HCX PL APO CS 20X/0.7NA long working distance physiology objective (Leica). The laser was centered at 850 nm to excite AF and Hoechst 33342 fluorescence. TPEF signals were collected in epifluorescence configuration, split with a dichroic mirror, through multiphoton bandpass filters (TPEF = 525/50 or 500–550 nm; epifluorescence = 585/40 or 565–605 nm; Leica), and guided onto a

non-descanned photomultiplier tube (PMT) detector (NDD; Hamamatsu, Bridgewater, NJ). Images were collected as Z-stacks (xyz, 600 Hz, bidirectional) using 512×512 -pixel or 1024×1024 -pixel resolution and $16 \times$ line averaging.

Images were captured using LAS AF (Multiphoton; Leica), and analyzed with Velocity 5.4.1 (PerkinElmer, Waltham, MA) or LAS AF Lite 2.2.1 (Leica). Image frames at a depth c.a. $40 \mu\text{m}$ to $60 \mu\text{m}$ from the UV surface; for example, the face of the wedge abutting the anterior chamber was selected to represent each wedge. Images were cropped, resized, and fit into figures using Photoshop CS5 (Adobe, San Jose, CA).

RESULTS

AF

The TM (Fig. 2A) was located between cornea and ciliary muscle. A distinctive autofluorescent structural configuration corresponding to UV (Fig. 2B), CS (Figs. 2C, 2D) and juxtacanalicular (JCT) (Fig. 2E) meshwork was seen. This varying structural configuration provided landmarks for tissue location and depth. The AF signal from the innermost UV meshwork showed slender branching beams separating large gaps (Fig. 2B). The CS meshwork was plate-like with wide fluorescent bands separating pores lacking AF (Figs. 2C, 2D). These pores varied in size from less than $10 \mu\text{m}$ to greater than $20 \mu\text{m}$. In contrast, the gaps of the UV meshwork were

typically greater than $40 \mu\text{m}$ in diameter. In the JCT, arrays of fine autofluorescent fibers were seen (Fig. 2E).

Viability

Calcein AM-positive cells were prevalent, and PI-positive cells were few in the CS meshwork (Fig. 1B), and the JCT (data not shown). Cellularity was found to be limited to regions of AF, which was reduced in the UV meshwork (Fig. 2B, compare with Figs. 2C–E). This reduced cellular density was reflected in the Calcein AM and PI labeling. The staining pattern of Calcein AM in individual, Calcein AM-positive cells was similar throughout all layers (data not shown). In tissues incubated with detergent, no Calcein AM-positive cells were found; PI-positive cells were prevalent (Fig. 1A).

Indirect Epifluorescence

We focused imaging to the CS meshwork (40 – $60 \mu\text{m}$ deep to the UV meshwork) (Figs. 3, 5, 7, 8) as the multimodal TPEF combination of indirect epifluorescence, intravital dye fluorescence, and AF signals were optimally captured in this region. Apart from optimizing imaging, selecting a standardized region provided consistency and facilitated detection, localization, and direct comparison of labeled (α -SMA, myocilin, type-IV collagen, and FN), and unlabeled (autofluorescent) markers in the tissue. Select examples of positive labeling in the UV, CS, and JCT meshwork (Figs. 4, 6) have also been shown to allow imaging in these different regions to be compared.

α -SMA

With exposure to the vehicle control for TGF- β 1 (serum-free DMEM), GFAP (Figs. 3A–C) and α -SMA (Figs. 3D–F) staining in the CS meshwork was faint and barely above background. When tissue was exposed to TGF- β 1, fluorescence labeling of α -SMA was intense and widely distributed (Figs. 3G–I). Intensity of α -SMA labeling was much greater in TM exposed to TGF- β 1 than the control vehicle alone. Positive α -SMA staining in the ciliary muscle region was characterized by short, wavy streaks (Figs. 3J–L, asterisk). By contrast, positive α -SMA in the TM was diffused and coincided with autofluorescent beams (compare Figs. 3I and 3L). Figure 4 shows increased α -SMA labeling in the UV, CS, and JCT meshwork compared with vehicle controls.

Myocilin

Following exposure to Dex, myocilin was widely expressed in the basement membrane of the CS meshwork (Figs. 5D–F, 5J–L) where it lined autofluorescent structures (Figs. 5E, 5L). Myocilin labeling was most intense in regions immediately adjacent to pores (Fig. 5E inset). When tissue was exposed only to vehicle control, no positive TM staining was seen (Figs. 5A–C, 5G–I). Positive myocilin immunostaining was similar in tissue from separate donors (compare Figs. 5D–F and 5J–L). When tissues were fixed without glutaraldehyde (formalin only), the staining pattern featured a more cytosolic distribution, and less extracellular localization (Fig. 6). High-magnification optical sectioning to capture individual cells showed cytosolic, perinuclear myocilin labeling among autofluorescent structures of the UV (Figs. 6A–C), CS (Figs. 6D–F), and JCT (Figs. 6G–I) meshwork. The intracellular labeling looked similar in different TM regions, reflecting effective primary and secondary antibody tissue penetration.

FIGURE 2. AF features of the human TM. (A) En face optical section through ciliary muscle (*top*: dense horizontal fiber AF), TM (*middle*: branching AF; between hash lines) and cornea (*bottom*) on their anterior chamber side. Hoechst 33342 labeling shows nuclei. The image slice captures the TM in oblique section, revealing the autofluorescent branching beams of the UV meshwork above, and denser structural AF and cellularity of the CS meshwork below; $\times 20$. Bar equals $50 \mu\text{m}$. (B–E) Higher power sections through various depths at asterisk; all $\times 63$. Bar equals $25 \mu\text{m}$. (B) UV meshwork ($5 \mu\text{m}$ deep). Nuclei were associated with branching autofluorescent beams. (C) CS meshwork ($40 \mu\text{m}$ deep) had more cell nuclei, wider autofluorescent beams, and pore-like structures. (D) Deeper CS meshwork ($50 \mu\text{m}$ deep) had a denser structural and nuclear organization than (C). (E) JCT meshwork ($75 \mu\text{m}$ deep) possessed a dense cellular arrangement among arrays of autofluorescent fibers.

FIGURE 3. DME (A–F) compared with TGF- β 1-induced α -SMA (G–L) expression in the tissue. The anterior (A–D) and posterior (J–L) CS meshwork was imaged after incubation with anti-GEAP (A–C) or anti- α -SMA (D–L) antibodies. Only faint α -SMA labeling above background was seen in tissue treated with DME (*arrows*: D, compared with A). Multichannel images of anti- α -SMA antibody labeling (D, G, J), and AF (E, H, K) were merged (F, I, L). Ciliary muscle bordered the posterior TM (*asterisk*: J–L) and showed wavy fluorescence. All tissue shown (A–L) was from the same donor. Bar equals 25 μ m.

FIGURE 4. TGF- β 1-induced α -SMA expression in the UV, CS, and JCT meshwork (D-F) compared with DME (scant red stain; A-C). Multichannel images of anti- α -SMA antibody labeling (*left column: red*) and AF (*middle column: green*) of the UV (A, D), CS (B, E), and JCT (C, F) were merged in the two far right columns showing en face and orthogonal views. α -SMA labeling associated with AF structures. Dotted lines indicate the depth of image capture: 15 μ m (A, D), 45 μ m (B, E), and 75 μ m (C, F) deep to the UV meshwork surface. Imaging gain was adjusted to compensate for depth-related fluorescence intensity loss and matched for frames of identical depth. All tissue shown (A-L) was from the same donor. Bar equals 25 μ m.

FIGURE 5. Dex induced high myocilin expression (D–F, J–L) in the TM compared to vehicle controls (A–C, G–I), as shown for separate donors (*donor 1*: A–F; *donor 2*: G–L). Multichannel images of antimyocilin antibody labeling (A, D, G, J) and AF (B, E, H, K) were merged (C, F, I, L). Inset in (F) shows $\times 2$ zoom of area marked by *asterisk*. Gain settings for vehicle and Dex-treated tissue were adjusted and matched to minimize background fluorescence. Myocilin fluorescence was particularly intense at the borders of pores and beams. Bar equals 25 μm .

FIGURE 6. Perinuclear myocilin labeling was seen regardless of tissue depth. Intracellular expression of myocilin was induced by Dex 250 nM. Multichannel images of antimyocilin antibody labeling (*left column: red*), Hoechst 33342 labeling (*green nuclei*), and AF (*middle column: green*) were merged, as shown in the two far right columns (*en face and orthogonal views*). AF cues guided identification of UV (A–C), CS (D–F), and JCT (G–I) regions. Dotted lines indicate optical slice depth relative to the UV meshwork surface: 20 μ m (A–C), 45 μ m (D–F), and 75 μ m (G–I). Imaging gain was adjusted to compensate for depth-related fluorescence intensity loss and matched for frames of identical depth; formalin fixation. Bar equals 10 μ m.

Type-IV Collagen

Indirect epifluorescence of type-IV collagen revealed regions of positive staining with reference to autofluorescent structures (Fig. 7). Tissue anti-GFAP antibody labeling was scant, punctate, and random (Figs. 7A–C). Anti-type-IV collagen antibody labeling displayed a distinct pattern, coinciding with the basement membrane region of autofluorescent structures (Figs. 7D–F). The most intense type-IV collagen labeling was located at the edge of autofluorescent structures bordering pores (Fig. 7E, inset), resembling the region labeled for

FIGURE 7. Indirect immunofluorescence (IF) labeling of type-IV collagen. Tissue was incubated with anti-GFAP (A–C) or anti-type-IV collagen (D–F) antibodies. Multichannel IF (A, D; *red*) and AF (B, E; *green*) were imaged in parallel in the CS meshwork and merged (C, F). (F) Inset shows 3 $\times$ zoom of area marked by an *asterisk*. Bar equals 25 μ m.

FIGURE 8. Indirect IF labeling of fibronectin (FN). (A–F) Tissue was incubated with anti-GFAP (scant red labeling; A–C) or anti-FN (*red*; D–G) antibodies. Multichannel IF (A, D) and AF (*green*; B, E) were imaged in parallel in the CS meshwork and merged (C, F). *Arrows* indicate pores as suggested by the signal void within the AF. Bar equals 25 μ m. (G) A $\times 20$ maximum projection image of optical sections from 100 μ m of tissue. Bar equals 100 μ m.

myocilin (compare with Fig. 5). This distribution indicated basement membrane localization for both markers.

FN

FN was widely distributed throughout the TM (Fig. 8). In a similar region to that of Figures 1, 3, and 5 (40–60 μ m deep to the UV surface), TPEF revealed pores among autofluorescent structures (Figs. 8B, 8E, *arrows*). Anti-GFAP antibody labeling was scant, punctate, and random (Figs. 8A–C). Anti-FN antibody labeling was widely distributed in the form of multiple 2- to 5- μ m curvilinear regions of intense positive fluorescence among autofluorescent structures (Figs. 8D–F). A lower power, projection view of FN staining through 100 μ m of the TM revealed a uniform and reticular-like distribution amidst the AF (Fig. 8G).

Nuclear Localization

There was variation in the distribution of type-IV collagen, fibronectin, α -SMA, and myocilin epifluorescence labeling with respect to cells, as represented by Hoechst 33342 nuclear fluorescence (Fig. 9). Type-IV collagen linearly outlined autofluorescent trabecular beams, but did not colocalize with the cores of beams, or associate with nuclei (Fig. 9A). Similarly, FN was distributed in close proximity to autofluorescent beams, but did not colocalize with beams (Fig. 9B). While FN was not generally associated with nuclei, its labeling was occasionally observed within 5 μ m of nuclei (Fig. 9B, *cross*). α -SMA staining was observed in close proximity to nuclei and its distribution was distinctly perinuclear (Fig. 9C). Myocilin staining was mostly extracellular and closely associated with autofluorescent structures (Fig. 9D, *asterisk*); although, it was also seen adjacent to nuclei (Fig. 6; also Fig. 9D, *arrows*).

FIGURE 9. Association of Hoechst 33342-labeled nuclei with expressed proteins in merged multichannel IF (red), AF (green), and Hoechst (green nuclei) images. (A, B) Type-IV collagen and fibronectin staining associated primarily with autofluorescent structures rather than nuclei. (B) Cross indicates fibronectin near a nucleus (Hoechst) although its association was with the autofluorescent extracellular matrix. (C) Cytosolic α -SMA was perinuclear. (D) Myocilin staining was located along an autofluorescent structure (asterisk) and also perinuclearly (arrows). Figures (A) and (C) were captured in the UV meshwork; (B) and (D) in the CS meshwork. Bar equals 10 μ m.

DISCUSSION

We are developing and validating an *in situ* model of the human TM, in which to study tissue biology and pathogenic mechanisms. They have used TPEF deep tissue optical sectioning, and a multimodal approach combining indirect epifluorescence, intravital dye, and AF imaging. Traditional histological methods requiring embedding and physical sectioning were unnecessary. By this approach, tissue induction, and expression of proteins characteristic of TM cells and pertinent to glaucoma, has been described. Specific intracellular and extracellular epitopes were identified deep in the tissue, and relative to the ECM and cells. Induction and expression of α -SMA and myocilin by TGF- β 1 and Dex, respectively, both important to glaucoma, could be localized *in situ*. Likewise, characteristic TM ECM markers of type-IV collagen and fibronectin were identified.^{1,2,4,5,7,8,12,14,15} This study provides unique views and insights into the possibility of identifying and exploring important biological interactions in the TM.

We could resolve associations between tissue architecture, ECM proteins, and cells *in situ*. TPEF localization of tissue markers was consistent with foregoing reports based on cell culture, electron microscopy, and immunohistochemistry studies.^{3,7,20–32} Type-IV collagen, fibronectin, and myocilin were associated with autofluorescent structures in a region consistent with basement membrane.^{3,7,20–22} The myocilin findings agree with prior reports of the protein's localization to intracellular compartments,^{13,23–29} and extracellular association with long-spacing collagens and sheath material surrounding elastin.^{3,23} We postulate that AF in TM structures originates in the cores of trabecular beams comprising structural ECM

proteins such as elastin and type-I and type-III collagen^{22,30–32} that are endogenous fluorophores.³³ The TGF- β -induced α -SMA intracellular localization¹⁶ is relevant to TGF- β -mediated tissue changes. This approach potentially makes it possible to study these important markers *in situ*, as well as other entities such as proteoglycans³⁴ that otherwise are lost to harsher immunohistochemistry processing.

Overall, we found the CS meshwork to be optimal for image characterization of tissue structure, and intracellular and extracellular markers: a significant density of cells was present; ECM structures were distinctive; epifluorescence and AF signals were optimal; tissue depth here did not compromise antibody or laser penetration; and there was good consistency of results across tissues and donor eyes. By comparison, fluorescence signals were of lower intensity, and less distinct, deeper in the JCT and inner wall of Schlemm's canal; images here were still informative, however, as reduced fidelity could be compensated for, in part, by appropriate adjustments such as increasing acquisition gain.

Indirect immunofluorescence of whole unsectioned tissue may be hampered by poor antibody penetration,^{35–39} nonspecific binding,^{40–48} and diminished laser power with deeper tissue imaging. The antibody penetration was improved by longer incubations, coupled with nonionic Triton X-100 detergent permeabilization. Five percent Triton X-100, which has been used in other deep tissue imaging studies (e.g., spinal cord),⁴⁹ was an appropriate concentration for TM deeper tissue permeabilization. We are now determining if it is appropriate for labeling cytosolic and membrane-associated markers, such as actin and associated proteins. They also found that different fixation (e.g., without glutaraldehyde) could alter staining patterns and perhaps even antibody penetration. These effects are likely unique to antibody type and need to be matched to the epitope under study (e.g., extracellular versus intracellular) and experimental conditions.

Myocilin staining in the UV and CS meshworks was similar when adjusted for differing laser penetration with depth; however, even with compensation myocilin staining was harder to see in the JCT. This could be due to a combination of issues pertaining to laser penetration, antibody penetration, variable myocilin expression, or some combination of the above. Immunoelectron microscopy has previously confirmed the presence of myocilin in the JCT.⁵⁰ We have tried different antimyocilin antibodies to similar effect. In the present article, JCT-localized antibody labeling of α -SMA and fibronectin was demonstrated.

Localization in the z-axis was found to be nonprecise, resulting in the apparent colocalization of noncompatible signals, as was the case for the apparent colocalization of fibronectin with nuclei (Fig. 9B, cross). In this case, it is very likely that the indirect fluorescence of labeled fibronectin is above or below the nucleus. Current multiphoton systems feature submicron resolutions in the x-y plane, but z-resolution is 10 times less, affecting the accuracy of localization in the z-axis. The exact location of the signal is determined by adjusting optical section imaging until peak fluorescence is seen.

This method currently has clear limitations. First, fluorescence intensity diminishes with depth as the JCT and the SC inner wall are approached. If the methodology could improve, such that the changes in the JCT and inner wall areas could be visualized at a similar resolution as the CS meshwork, this technique would be a more valuable tool for discovery in the field of anterior segment glaucoma research. Another limitation of this technique is that the studies previously and currently performed using this model are not under physiological flow conditions. If the model could be adapted to accommodate such conditions, this technique would be a powerful tool for

studying aqueous humor outflow dynamics and its regulation by cells and matrix components.

It will be an advance if human donor eye tissue can be salvaged and harnessed for glaucoma research, utilizing a relatively scarce, but quality resource. The human donor tissue, we report, was viable enough to be induced to express specific proteins. This is not surprising given the tissue was suitable for human corneal transplantation. Such tissue can also be used to establish primary TM cells in culture⁵¹ and maintained in perfusion organ culture over prolonged periods.^{52,53} As eye bank donor tissue may be compromised during storage and processing, it was important to ascertain tissue quality, as described in the Methods section. The screening included performing cellular viability and cell death intravital dye co-labeling in situ (Fig. 1).^{18,19} During quality screens, suboptimal tissue could be identified and excluded from further analysis.

This multimodal approach potentially allows biological interactions to be analyzed within the 3D architecture of the aqueous drainage tissue. This analysis is possible by direct observation, and with a level of detail expected of in vitro, but not in vivo studies. Unlike in vitro models, the 3D tissue context is preserved. Importantly, the original relationship between TM and SC is retained. This provides a way to study the TM-SC tissue complex, which has the highest fluid drainage resistance in the outflow tract. The proposed use of human donor tissue can be studied in a way that is accessible compared with fresh, whole human eyes that are scarcer and more expensive.

These findings indicate that a variety of disease-associated markers can be localized within the original 3D environment of the human TM at the submicron scale. Characterization markers for TM cells, that are also relevant to glaucoma pathogenesis, could be identified in situ using the approach and tissue that have been described. These findings are consistent with prior in vitro, electron microscopy and immunohistochemistry observations, offering cross-validation between this system and prior models. The proposed system is not expected to replace well-established models; rather, it allows research questions of a different kind to be addressed. These data suggest that molecular mechanisms of IOP regulation, glaucoma pathogenesis, and pharmacology can be explored by direct observation in such a system. We intend to exploit this in future studies.

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