Analyzing Live Cellularity in the Human Trabecular Meshwork

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PURPOSE. To directly visualize the live cellularity of the intact human trabecular meshwork (TM) and quantitatively analyze tissue viability in situ.

METHODS. Human donor corneoscleral rims were sectioned immediately before intravital dye incubation to label nuclei (Hoechst 33342 & propidium iodide [PI]); cytosol (CellTracker Red CMTPX, calcein AM); and membranes (octadecyl rhodamine B chloride [R18]), followed by 2-photon microscopy. Viability was assessed by counting cells in tissue colabeled with PI and Calcein AM. Some tissues were exposed to Triton X-100 to establish dead tissue controls. Fresh postmortem eyes (within 48 hours of death) represented viable tissue controls. Tissues with live cellularity exceeding 50% were considered viable.

RESULTS. Hoechst nuclear labeling was seen throughout the TM, among the autofluorescent beams, plate-like structures and fibers of the meshwork, and within tissue gaps and pores. CellTracker-labeled live cells were attached to autofluorescent TM structures and filled corneoscleral meshwork pores. R18-labeling revealed the membrane distributions of interconnected cells. Calcein-positive cells were visible in all TM layers, but not in tissues killed by Triton X-100 exposure. Dead control tissues showed PI staining in the absence of Calcein-positive cells. Two-thirds of the standard donor tissues we received possessed viable TM, having a mean live cellularity of 71% ($n = 14$), comparable with freshly postmortem eyes (76%; $n = 2$). Mean live cellularity of nonviable tissue was 11% ($n = 7$).

CONCLUSIONS. We have visualized and quantified the live cellularity of the TM in situ. This provided unique perspectives of live cell-matrix organization and a means of assaying tissue viability. (*Invest Ophthalmol Vis Sci.* 2013;54:1039-1047) DOI: 10.1167/jovs.12-10479

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Cells in the trabecular meshwork (TM) play pivotal roles in regulating aqueous humor drainage, but the exact nature of cellular regulation remains poorly understood.¹⁻⁷ While cell culture studies provide some clues to TM cellular regulation, in vitro models lack the unique 3-dimensional (3D) organization of tissue, making it challenging to directly correlate cell and tissue function. Such correlation may be possible, however, if live cells could be observed directly and in situ within their unique, 3D tissue context.

In situ, the TM has an intricate 3D configuration that is subject to modulation by cells and neighboring tissues such as the ciliary muscle.^{5,7-12} The structure of the TM varies from slender branching beams in the uveal meshwork, to perforated sheets in the corneoscleral meshwork, to a fine fiber network in the juxtacanalicular meshwork (JCT).^{13,14} This structure, which can be imaged by autofluorescence (AF) microscopy,^{13,15} represents the TM's extracellular matrix (ECM) that supports cells and the function of the aqueous drainage tract.

We are developing a novel tissue model in which biological interactions can be directly analyzed in situ within the 3D human aqueous humor drainage tract. We have applied two-photon excitation fluorescence microscopy (TPEF) to image the TM within human donor corneoscleral rim tissue retained from corneal transplantation.^{13,15-22} Deep tissue imaging and optical sectioning of viable tissue is possible without the fixation and processing required for conventional histology that renders tissue nonviable.

Intravital dyes are nontoxic fluorescent labels that selectively stain cells and subcellular compartments, allowing their direct visualization by TPEF. TPEF exploiting multimodal approaches of AF, indirect epifluorescence, and intravital dye fluorescence can be used to visualize cells and protein expression within the 3D tissue.

In the present study, we have used intravital dye imaging to characterize the 3D organization of live cells within the human aqueous drainage tissue. Here, live cells were visualized with reference to autofluorescent TM structures that provided localization information in situ. CellTracker Red CMTPX (Life Technologies, Carlsbad, CA) was used to specifically label cytosolic spaces; octadecyl rhodamine B chloride (R18) labeled the plasma membrane. Live and dead cellularity within the TM of postmortem donor tissue was assessed by live/dead dye colabeling and software-assisted quantitative analysis.

MATERIALS AND METHODS

Human Donor Tissue. Surgeons of the Doheny corneal service provided residual human donor corneoscleral rim tissue after grafting. Corneal grafting typically occurred within 6 days postmortem.¹⁵ Additionally, we obtained fresh postmortem eyes within 48 hours of death to serve as viable control tissue. 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, we could not obtain specific information on the donor tissues apart from date of grafting. Donor tissues were received

right after surgery, maintained at 4°C in transport media (Optisol GS; Bausch & Lomb, Rochester, NY), and processed immediately after receipt. The central cornea had been removed, leaving the TM and Schlemm's canal (SC) drainage tissue intact. Prior to labeling, tissues were placed with the TM side up in transport media (Bausch & Lomb) on a glass dish and segmented into 8 to 12 wedges. Tissues were screened for viability as previously described.¹⁵

Intravital Dyes. Hoechst 33342 (nuclear label); octadecyl rhodamine B chloride (R18; membrane label); CellTracker Red CMTPX (cytosolic label); calcein AM, (cytosolic label and vitality dye); and propidium iodide (PI; labels dead nuclei) were purchased from Life Technologies. Hoechst 33342 is a widely used nuclear dye that permeates all membranes and intercalates between nucleotides in DNA.^{37,38} Hoechst 33342 was used at 40 ng/mL to 1 µg/mL in PBS, or in serum-free low glucose DME (lg-DME; Mediatech, Washington, DC) and occasionally in combination with other dyes, for 15 minutes to overnight at room temperature (RT) or at 37°C and 8% CO₂. CellTracker Red (Life Technologies) crosses cell membranes and become fluorescent in the presence of esterases.^{23–28} Further enzymatic action by glutathione-S-transferase renders the molecule hydrophilic and unable to exit cells, thus providing a live cell cytosolic label. CellTracker Red (Life Technologies) was used at 25 µM in serum-free lg-DME for 45 minutes at 37°C and 8% CO₂. CellTracker Red-stained wedges were placed in CellTracker Red-free, serum-free lg-DME and incubated for 30 minutes at 37°C and 8% CO₂, then washed three times in PBS at RT ($n = 5$). R18 labels plasma membranes.^{29–32} R18 was used at 200 µM to 2 mM in PBS for 45 minutes at 37°C and 8% CO₂. R18-stained wedges were then washed three times with PBS at RT ($n = 3$). TPEF was then performed. No fixation, embedding, or permeabilization of the tissue was needed.

Viability Colabeling with Calcein AM and PI. Upon receipt, donor tissues were sectioned as described above. One section was designated a control for dead tissue and incubated with 0.2% nonionic surfactant (Triton X-100 [TX-100]; EMD Millipore, Billerica, MA) in PBS (pH 7.4) for 30 minutes at room temperature. The remaining sections were left in PBS at room temperature during the incubation. TX-100-treated (EMD Millipore) and untreated wedges were incubated with 0.3 µM calcein AM^{33–35} and 1 µg/mL PI^{37–39} and/or 1 µg/mL Hoechst 33342 for 30 minutes at 37°C and 8% CO₂. TPEF was performed immediately after incubation.

Manual Cell Counting. Live and dead cells were counted through entire *z*-stacks using 8-µm steps through the *z*-plane. For example, if a *z*-stack comprised optical sections at 1-µm intervals, only cells from every eighth frame were counted. This was done to minimize the chance of double-counting cells. Depending on the concentration of PI used, PI sometimes labeled the nuclei of living cells, albeit more dimly compared with dead nuclei. To obviate this, PI concentration was appropriately titrated in assays, down to a minimum of 1 µg/mL. Treatment of tissue with TX-100 (to kill cells; EMD Millipore) showed positive PI labeling and negative calcein labeling. This confirmed PI labeling of dead cells in the absence of calcein positively labeled live cells, wherein calcein-negative, PI-positive cells were considered dead cells. Percentage of live cellularity was determined by calculating the ratio of calcein-positive cells to the total number of cells (the sum of calcein-positive live cells and PI-positive dead cells).

TPEF Setup. A confocal microscope system (Leica TCS SP5 AOBs MP; Leica Microsystems, Heidelberg, Germany) coupled to a multiphoton laser (Chameleon Ultra-II; Coherent, Santa Clara, CA) was used. Tissues were imaged TM-side down on a glass-bottom microwell dish. Incident light was focused, and emitted signals collected, with an inverted glycerol objective (HCX PL APO CS 63×/1.3 NA; Leica Microsystems).

Imaging. TPEF signals were collected in epifluorescence configuration, split with dichroic mirrors, and guided through multiphoton bandpass filters (TPEF = 525/50 nm [Leica Microsystems] and epifluorescence = 635/90 nm [Chroma, Bellows Falls, VT]) to a nondescanned photomultiplier tube 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× line averaging. The whole length of the 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 (NDD; Hamamatsu). These imaging settings allowed AF of fine structures of the TM such as ECM beams, plates, and fibers to be seen.¹³ AF excitation was at 850 nm. Cells and subcellular structures that were labeled with intravital dyes were characterized with reference to autofluorescent TM structures. Images were analyzed with microscope data software (LAS AF Lite 2.2.1 [Leica Microsystems] and Imaris 7.3.0 [Bitplane, Zurich, Switzerland]); cropped, resized, and fit into figures using a graphics editing program (Photoshop CS5; Adobe, San Jose, CA). Surface mapping polygonal reconstruction and 3D manipulation was performed in Imaris (Bitplane).

RESULTS

Autofluorescent Structure

The AF structure of the TM varied with depth in the tissue. Figure 1A shows slender AF beams (arrows) with large intervening gaps (asterisks) in the uveal meshwork; AF plate-like structures (Fig. 1B, double-bar crosses) with smaller pores (asterisks) were seen in the corneoscleral meshwork; fine arrays of AF fibers were seen in the JCT (Fig. 1C). Hoechst 33342-DNA nuclear labeling was present throughout the TM. Labeled nuclei associated with uveal meshwork autofluorescent beams. In the corneoscleral meshwork, labeled nuclei associated with the plate-like structure and pores. In the JCT, labeled nuclei were seen among autofluorescent fiber arrays. In 2-dimensional (2D) 0.06 mm² optical slices, nuclear density increased with depth, from 69.8 ± 13.8 nuclei per optical slice of the uveal meshwork (Fig. 1A), to 103 ± 26.1 nuclei in the corneoscleral meshwork (Fig. 1B) and 98.2 ± 29.8 nuclei in the JCT (Fig. 1C).

Cytosolic and Hoechst 33342–Nuclear Labeling

Figure 2 shows CellTracker cytosolic labeling (Life Technologies) in live cells (red) distributed throughout the TM. CellTracker (Life Technologies) labeling also showed some affinity for autofluorescent fine fibers (yellow). The labeled cells were in gaps between uveal beams and corneoscleral meshwork pores. In the JCT, the distribution of CellTracker-labeled (Life Technologies) cells was sheet-like among autofluorescent fiber arrays (Fig. 2G). CellTracker (Life Technologies) labeling was often punctuated by tiny granular-appearing signal voids probably representing unlabeled organelles (thin arrows).

With Hoechst 33342 and CellTracker (Life Technologies) colabeling, the nuclear labeling was inconsistent, especially with tissue depth. Where Hoechst labeling was present, the nuclear labeling was distinct from CellTracker cytosolic labeling (Life Technologies) as the labels did not coincide. In cells with unlabeled nuclei, nuclear location was still evident by oval-shaped voids within the CellTracker-labeled cytosol (Life Technologies; Fig. 2, broad arrows).

Figure 3 shows clusters of Hoechst 33342-labeled nuclei inside corneoscleral meshwork pores (Figs. 3A–C). Correspondingly, CellTracker-labeled (Life Technologies) cells were densely packed in corneoscleral meshwork pores (Figs. 3D–H). Orthogonal reconstruction of serial sections revealed layers of CellTracker-labeled (Life Technologies) cells interspersed among stratified corneoscleral meshwork plates (Figs. 3E–G). Serial sections 1 µm apart show cell associations within a pore

FIGURE 1. Autofluorescence and nuclear labeling of human corneoscleral donor rim tissue. (A) Optical sections through the uveal meshwork (5- μ m depth; from uveal surface). (B) Optical sections through the corneoscleral meshwork (45- μ m depth). (C) Optical sections through the juxtacanalicular meshwork (65- μ m depth) accompanied by orthogonal reconstructions (right). (D) 3D representation of fluorescence fibers and Hoechst-labeled nuclei. The uveal meshwork aspect faces the reader. Scale bar: 25 μ m. Hash marks: sectional depth in orthogonal. Asterisks: gaps in uveal meshwork; pores in corneoscleral meshwork. Arrows: autofluorescent beams. Double-bar crosses: autofluorescent plates.

(24–29 μ m) and the beginnings of cells in adjacent connecting pores (30 μ m, Fig. 3H).

Figure 4 illustrates a cell with nucleus (arrowhead) wrapped around a bundle of trabecular fibers (arrow) and interacting with a second bundle of fibers (asterisks) across a 5- μ m gap in the uveal meshwork. The 3D image is a reconstruction of a 55 μ m $\times$ 35 μ m $\times$ 15 μ m volume. A movie is available of the observed cell, reconstructed in 3D, that rotates the cell in 360 degrees (see Supplementary Material and Supplementary Movie S1, <http://www.iovs.org/lookup/suppl/doi.10.1167/iovs.12-10479/-/DCSupplemental>).

R18 Membrane Labeling

R18 fluorescently labeled cell membranes and revealed cell borders as shown in Figure 5 (arrows). Individual R18-labeled cells were more distinct in the uveal meshwork (Fig. 5A) than in the corneoscleral (Fig. 5B) and JCT meshworks (Figs. 5C, 5G), where neighboring cells appeared to blend into each other. In these cases, Hoechst 33342 nuclear colabeling helped single out cells among clusters or sheets of R18-labeled cells.

Live/Dead Labeling: Calcein AM and PI

Nuclei labeled by Hoechst 33342 were not necessarily alive, as shown in Figure 6 in which PI identified dead cells.

Figure 7 shows PI and calcein colabeling for live/dead analysis. The colabeling strategy was first tested in dead control tissue derived by exposure of standard postmortem (6-day) donor tissue to TX-100 (EMD Millipore) (Figs. 7C, 7E, 7D). In these dead controls, no calcein-positive labeling whatsoever was observed. Instead, extensive PI-positive nuclei were identified in all layers of the tissue. Calcein-positive cells were numerous in tissues from the same standard postmortem eyes not exposed to detergent. Sometimes weak PI-positive labeling was seen among calcein-labeled cells (Fig. 7A; yellow colocalization of green calcein and red PI labeling). We

considered weak PI-labeling in the presence of calcein-positive colabeling in the same cell to represent false-positive PI labeling. The converse scenario of PI positivity without calcein during colabeling represented true cell death, as typically seen in the detergent-killed tissue in which calcein labeling was absent. The scenario of PI-positive, Calcein-negative colabeling was scant in the good quality standard postmortem tissue we tested (Figs. 7B, 7E, 7H). The same observation was made in fresh, 48-hour postmortem eyes that we obtained and studied as viable control tissue (Figs. 7A, 7D, 7G). Tissue in which PI-labeling predominated in the absence of calcein labeling was considered nonviable.

Calcein-positive cells were brightly fluorescent to the point where cytosolic fluorescence masked autofluorescent beams (Figs. 7A, 7B). The fluorescent cytosolic labeling became less intense with tissue depth (Figs. 7D, 7E, 7G, 7H).

In Figure 8, calcein cytosolic labeling of viable cells revealed diverse cellular morphology. Some cells appeared spread, with features reminiscent of lamellipodia (arrowheads), while others were more rounded (Figs. 8E, 8F). Long cytosolic extensions were seen (Fig. 7A, asterisk). Cells were seen in contact or closely associated with neighboring cells and could be distinguished from each other (Figs. 8E–H).

Quantitative Viability Analysis

To quantify live cellularity, calcein-positive cells that were considered alive were identified and manually counted as illustrated in Figures 8E, 8G, and 8H. PI-positive cells that were calcein-negative were counted as dead.

We arbitrarily defined viable tissues as having a proportion of live cells to all cells (live and dead) exceeding a cutoff of 50%. Tissues with a lower proportion than this were considered nonviable. We applied this definition to the live cellularity analysis of fresh sub-48-hour postmortem tissues that we used as viable control tissue. We calculated a live

FIGURE 2. Live cell CellTracker-cytosolic labeling. (A–C) Uveal meshwork. (D–F) Corneoscleral meshwork. (G–I) Juxtacanalicular meshwork. (A, D, G) Merge of CellTracker red Hoechst 33342 (green nuclei) labeling and autofluorescence (green fibers). Hoechst labeling was inconsistent in the presence of CellTracker colabeling (unlike Fig. 1), especially deeper in the TM. (B, C, E, F, H, I) Detail of individual cells located between beams in the uveal meshwork (B, C), in corneoscleral (E, F) meshwork pores, and among juxtacanalicular meshwork fiber arrays (H, I); magnification: $\times 2.5$. *Arrowheads*: Hoechst 33342-labeled nuclei. *Broad arrows*: CellTracker-labeled cells with unlabeled nuclei. *Thin arrows*: cytosolic signal voids. *Asterisks*: gaps between trabecular beams. *Double-bar crosses*: autofluorescent beams (green, or yellow due to some CellTracker affinity for fibers).

FIGURE 3. CellTracker- (red) and Hoechst 33342- (green ovals) labeled cells in corneoscleral meshwork pores. (A) Hoechst-labeled nuclei in a pore surrounded by autofluorescent fibers (green). (B, C) Orthogonal reconstructions of (A), vertical (B), and horizontal (C) showing cross-section of nuclei in a pore. Hash marks: depth of optical slice and X-Y positioning of cut. z-depth = 32 μm . (D) First of seven serial sections through CellTracker-labeled cells in a pore. (E) *En face* 3D reconstruction of serial sections. (F) Orthogonal view showing layers of cells amongst stratified autofluorescent plates (green). (G) Surface-mapped polygon reconstruction of orthogonal image showing at least six autofluorescent plates (green) in cross-section. (H) Serial optical sections 1 μm apart through cells in a pore. Left column: merge of CellTracker and Hoechst 33342-labeled cells and autofluorescence. Right column: autofluorescence and Hoechst 33342 labeling (inconsistent in colabeling with CellTracker). Dark ovals: unlabeled nuclei. Yellow: beams with coincident autofluorescence/CellTracker labeling. Hashed-boxes (E-G): location of serial sections in (D, H). Asterisks: connecting pores. Scale bar: 10 μm .

FIGURE 4. 3D reconstruction of CellTracker-labeled cell wrapped around a trabecular beam comprising fine ECM fibers. Serial rotation reveals the complex 3D interaction between cell and structural ECM. Arrows: cell body association with trabecular fiber. Arrowheads: Hoechst 33342-labeled nucleus. Asterisks: cell association with nearby trabecular fibers. Scale bar: 5 μm .

cellularity percentage of $76\% \pm 10\%$ (mean $\pm$ SD) in this viable control tissue as shown in Figure 9.

Live cellularity was analyzed in 21 individual donor rims representing the standard postmortem tissues we receive, as summarized in Figure 9. Live cellularity exceeded 50% in tissue from 14 eyes; this tissue was considered viable. Mean live cellularity in the viable tissue was $71\% \pm 14\%$. Live cellularity was less than 50% in tissues from seven eyes; these tissues were considered nonviable. Mean live cellularity in the nonviable tissues was $11\% \pm 13\%$.

Live cellularity was calculated as the percentage of cells counted in $246 \mu\text{m} \times 246 \mu\text{m} \times 100 \mu\text{m}$ 3D z-stack volumes per tissue. Mean cell count per z-stack volume was similar across the tissue groups: 366 ± 73 cells in viable control tissue; 429

FIGURE 5. R18 labeling in the human TM. R18 labeling (red) of cell membranes. (A) Uveal meshwork (z -depth = 25 μ m). (B) Corneoscleral meshwork (z = 50 μ m). (C) Juxtacanalicular meshwork (z = 75 μ m). (D–G): At higher magnification ($\times 2$) membranes of adjacent cells appear to blend together ([D, E], *hollow arrows*). Cells wrap around autofluorescent beams ([E, F], *beam fibers: double-bar crosses*). A lamellipodium-like structure is seen (*asterisk*). In the JCT, membrane labeling was continuous across sheets of cells (G) and nuclear labeling (*green ovals*) helped identify individual cells. *Arrows*: edge of cell membranes. *Scale bar*: 10 μ m.

± 181 cells in viable standard postmortem tissue; and 391 ± 111 cells in nonviable standard postmortem tissue. The number of calcein-positive cells in viable tissue was similar: 279 ± 21 cells in viable control tissue; 305 ± 131 cells in viable standard postmortem tissue ($P = 0.79$). The number of calcein-positive cells in nonviable tissue was 43 ± 51 .

DISCUSSION

We have applied intravital dye-assisted TPEF imaging to characterize the live cellularity of the TM in human donor corneoscleral tissue. Live cells and subcellular compartments

such as nuclei, cytosol, and cell membrane were visualized. Live cell organization and its variation through the depth of the tissue could be characterized in detail, including cellular associations with autofluorescent ECM structures. This provided unique 3D views that may be extrapolated to living tissue. Live/dead intravital dyes were exploited to quantify the live cellularity and viability of the TM using 3D quantitative image analysis. We found that the majority of standard donor tissue we received was viable and that the viability of this standard tissue was similar to that of fresh postmortem tissue analyzed within 48 hours of death.

Intravital dye labeling was useful as a tool for analyzing live cells in the postmortem TM, allowing cellular organization within the whole tissue to be assessed without traditional histological processing that irretrievably alters tissue. The intravital dyes we used penetrated deeply into the aqueous drainage tissue so that labeled cells were seen in all tissue depths from uveal to corneoscleral to JCT meshwork. Cells could be localized with reference to other cells and autofluorescent structures. Subcellular compartments such as nuclei, cytosol, and cell membrane could be labeled and distinguished from each other. This provided unique visualization of the TM.

The combination of optical sections, intravital dye labeling, and 3D reconstruction allowed specific visualization of cell-cell and cell-ECM associations in the TM. The inner TM is considered to have a porous structure with open channels through which aqueous humor flows toward the higher

FIGURE 6. Hoechst and PI labeling in the human uveal meshwork. (A) *Arrowheads*: location of Hoechst-labeled nuclei (*green ovals*) among autofluorescent beams. (B) PI-labeled nuclei (*red ovals*), indicating cell death. (C) Overlay of (A, B). Two Hoechst-labeled nuclei did not colabel with PI (nuclear colabeling in *orange*). *Scale bar*: 10 μ m.

FIGURE 7. Calcein and PI colabeling. (A–C) Human uveal tissue. (D–F) Corneoscleral tissue. (G–I) Juxtacanalicular tissue. (A, D, G) Viable control tissue comprising fresh sub-48-hour postmortem tissue ($n = 2$). (B, E, H) Standard postmortem tissue received after transplant surgery (typically 6 days postmortem; $n = 14$). Calcein-positive cells (green) were most brightly fluorescent in the uveal meshwork, but fluorescence diminished with depth in the corneoscleral meshwork (D) and JCT (G) due to decreasing laser penetration. Occasional PI-positive cells (red ovals) were seen amongst Calcein-positive cells. (C, F, I): Dead control tissue. Some standard postmortem tissue was incubated with Triton X-100 (detergent-killed) prior to calcein AM and PI colabeling. PI-labeled cell nuclei (red ovals) but not Calcein-positive cells were seen in detergent-killed tissue. Arrowheads: calcein-positive labels and PI-positive labels coincide. Asterisk: long cytosolic extension. Thin arrows: PI-positive nuclei. Scale bar: 10 μm .

resistance JCT.^{40–42} This notion is supported by AF imaging (Fig. 1).¹³ An intriguing finding was that live cells in the corneoscleral meshwork lined pore-like openings of ECM plates (Figs. 3, 9). Live cells appeared to wrap around the meshwork plates onto the inner walls of pores, in analogous fashion to cells wrapping around uveal meshwork beams. At times, cellular filling of pores appeared dense. We wonder if this organization has implications for understanding cellular regulation of resistance and outflow.

CellTracker (Life Technologies) localized to cytosolic and perinuclear cellular regions, allowing discernment of nuclei even in the absence of nuclear colabeling. While CellTracker (Life Technologies) had mild affinity for ECM elements such as fibers, this could be overcome by digitally subtracting ECM cross-staining using coincident AF signals as an analytical mask. Secondary images were then created in which only CellTracker-labeled (Life Technologies) cells were seen. Thus, cellular organization could be characterized with reference to other cells, beams, plates, fibers, and pores of the TM. While tiny signal voids in both calcein and CellTracker-labeled (Life Technologies) cells might be artifacts, we propose they represent nonlabeling cytosolic compartments. Both dyes require esterase cleavage to form fluorescent molecules, which may be compromised by suboptimal pH conditions in subcellular compartments such as lysosomes.

FIGURE 8. Detail of Calcein-positive cells in human TM. (A–H) Calcein- and PI-labeled cells in situ. (A) Single calcein-labeled cell (green) spreads across a gap between intersecting autofluorescent beams. Lamellipodium-like protrusions are seen (arrowheads). (B) Single spread cell with a larger cross-sectional area. (C) A calcein-positive cell wraps around an autofluorescent beam. Two neighboring PI-positive labeled cells (red nuclei) are seen. (D) A calcein-positive cell borders a gap between beams. (E) Three calcein-positive cells (labeled 1, 2, and 3) are wrapped around beams. Two PI-positive/Calcein-negative cells (red ovals) lie between the calcein-positive cells. (F) Three calcein-positive cells lined up on a uveal meshwork autofluorescent beam. (G) Clusters of calcein-positive cells (numbered) in the corneoscleral meshwork. Faint demarcation between cells represents the unlabeled cell membrane; this facilitated cell counting. (H) Ten cells in a sheet (numbered) in the JCT. Scale bar: 10 μm .

Calcein functioned similarly to CellTracker (Life Technologies) as a viability and cytosolic dye, with the exception that it did not label TM ECM elements. Like CellTracker (Life Technologies), calcein revealed associations between live cells and the ECM beams, plates, fibers, and pores of the TM. Unlike CellTracker (Life Technologies), Calcein yielded additional views of intricate details such as cell shape, membrane features resembling lamellipodia, and configuration, such as cell wrapping or conformation to autofluorescent beams. Borders between neighboring cells were better seen, making it possible to identify and count individual cells. Calcein labeling intensity was also much higher than that of the CellTracker (Life Technologies), to the point that it tended to mask AF features.

With R18 membrane labeling, it was hard to distinguish neighboring cells from each other as labeled membranes blended together. This difficulty could be overcome by nuclear stain colabeling. R18 labeling made it possible to see distinct membrane features such as filaments linking cells and complex lamellipodia-like structures. Unlike the cytosolic intravital dyes, the extent and variation of cell shape and size could be appreciated with R18 labeling. We speculate that R18-assisted TPEF could be applied to assessing in situ live cell-cell interactions and their disruption, such as might occur with pharmacological actin disruption.

Postmortem tissue viability was testable by quantitative live cellularity profiling using an intravital dye colabeling strategy. We tested this strategy in killed tissues and fresh postmortem tissues that served as dead and viable controls respectively. Using an arbitrary live cellularity cutoff of 50%, we found that two-thirds of our standard postmortem tissues were considered viable, having a mean live cellularity of 71%, similar to that of viable controls. Hence, the majority of our donor tissues retained good viability despite being maintained in artificial medium for several days postmortem. This tissue, therefore, should not be discarded as it retains the live TM in its original 3D configuration and is useful for research.

Our live cellularity analysis ought to be distinguished from conventional histological cell counting that does not specifically assay live cells or tissue viability. Figure 6 illustrates that

FIGURE 9. Quantitative analysis of live cellularity in the human TM. *Left:* Viable control tissue ($n = 2$). *Middle:* Viable standard postmortem tissue ($n = 14$). *Right:* Nonviable standard postmortem tissue ($n = 7$). Exclusively calcein-positive cells and PI-positive cells were counted within $246 \mu\text{m} \times 246 \mu\text{m} \times 100 \mu\text{m}$ tissue volumes. *White column:* mean number of calcein-positive cells. *Gray column:* mean number of PI-positive cells. *Whole column:* mean of total number of cells (calcein-positive cells plus PI-positive cells). Total cell count was not significantly different between groups ($P > 0.05$).

Hoechst 33342, a commonly used intravital nuclear dye, labeled both live and dead cells, which required PI labeling to be distinguished. Based on our analysis of dead and viable controls, PI labeling could be nonspecific, but calcein AM labeling was very specific for live cells. We addressed any ambiguity from PI labeling by carefully interpreting signals in control data, appropriately titrating PI concentration, and colabeling all tissues with calcein AM.

We are developing an ex vivo human model in which TM cell and ECM interactions can be probed in situ by TPEF.^{13,15,22} In practice, tissue viability could be screened using the methods we report prior to attempting any studies in situ. Our analysis suggests that two-thirds of posttransplantation donor tissue is viable, which could be rapidly and empirically confirmed before usage. Tissue preparation, staining, and multiphoton screening analysis can be completed within 2.5 hours. Software-assisted quantitative live cellularity profiling is then performed. We are now developing automated methodology to make viability screening more efficient and practical.

Intravital dyes provide interesting options for visualizing cells, subcellular compartments, and cellular function in situ. An additional property of CellTracker (Life Technologies) that was not exploited in this study concerns its retention in daughter cells. This could allow cell fate, division, and possible translocation through tissue to be analyzed in situ, providing tools to address questions of putative progenitor cell behavior and repopulation.

We see a role for combined modality imaging in an in situ human TM model we are developing. We have previously reported autofluorescence features and immuno-characterization of protein expression in the model.^{13,15} Intravital dyes allow direct live cell analysis, have good tissue penetration, require less optimization than immunolabeling, and obviate the need for tissue fixation, permeabilization, and histological sectioning. Both intravital and immunofluorescence labeling can be combined with autofluorescence or second harmonic generation visualization,^{13,15} providing versatile options for in

situ analysis. This, combined with readily accessible and viable human tissue, should yield new opportunities for studying the aqueous drainage tissue.

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