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 intravitreal 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 interconnect-ed 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/iovs.12-10479

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Materials and Methods

Human Donor Tissue. Surgeons of the Doheny cornea service provided residual human donor corneoscleral rim tissue after grafting. Corneal grafting typically occurred within 6 days postmortem. 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

**Intravitreal Dyes.** Hoechst 33342 (nuclear label); octadecyl rhodamine B chloride (R18; membrane label); CellTracker Red CMTPX (cytosolic label); calcine 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 unable to exit cells, thus providing a live cell cytosolic label. Percentage of live cellularity was determined by calculating the ratio of calcein-positive live cells and PI-positive dead cells. Live and dead cells were counted through 8-μm-steps throughout the entire z-stack using 8-micrometer stacks 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 chances of double counting of intravital cells. Depending on the concentration of PI used, PI sometimes labeled the nuclei of living cells, albeit more dimly (asterisks) were seen in the corneoscleral meshwork fine arrays of AF fibers were seen in the JCT (Fig. 1A). 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 μm² 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).

**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 μm² 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).

**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 = 655/90 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 16x 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.15 AF excitation was at 850 nm. Cells and subcellular structures that were labeled with intravitreal 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).
(24–29 μm) and the beginnings of cells in adjacent connecting pores (30 μm, 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-μm gap in the uveal meshwork. The 3D image is a reconstruction of a 55 μm × 35 μm × 15 μm 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, 7F, 7I). 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) Juxtacanicular 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: ×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 XY 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 oval: 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% ± 10% (mean ± 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% ± 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% ± 13%.

Live cellularity was calculated as the percentage of cells counted in 246 μm × 246 μm × 100 μm 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
± 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...
resistance JCT.\textsuperscript{40–42} This notion is supported by AF imaging (Fig. 1).\textsuperscript{15} 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.

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...
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. 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. 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, 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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References


