Semi-automated vitality analysis of human trabecular meshwork

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Keywords: trabecular meshwork, multiphoton, calcein, viability, quantitative

Glucoma is associated with cell loss in the trabecular meshwork of the eye. Multiphoton microscopy aided by intravital dye labeling permits visualization of live and dead cells within the intact trabecular meshwork. We have developed a semi-automated method to quantify cellular viability within the human trabecular meshwork based on three-dimensional software-assisted tissue reconstruction and isosurface modeling. Live cellularity counts by the semi-automated method were obtained quickly and agreed with that of manual counting in the same group of tissues (1.6% group difference; n = 13), with counts in individual tissues showing a mean coefficient of variation of 10%.

Introduction

Primary open angle glaucoma (POAG) is a leading cause of irreversible blindness worldwide. The condition is associated with older age, elevated intraocular pressure (IOP), and reduced cellularity in the trabecular meshwork (TM).

The TM is a tissue that drains aqueous humor fluid from the eye and modulates IOP. Why TM cellularity should be reduced in POAG is unclear. Oxidative stress1,2 and gene mutations causing misfolded proteins in TM cells3-5 are among postulated pathogenic factors. It is possible that with reduced cellularity, TM function gradually fails, causing increased resistance to fluid drainage, elevated IOP, and glaucoma.

We have been developing approaches using 2-photon excitation fluorescence (TPEF) imaging to study glaucoma cellular pathogenesis in the TM in vivo6 and in tissue surgically removed from live patients with glaucoma. These approaches have been tested initially in a primary tissue model7-10 comprising human postmortem donor tissue containing the intact and viable TM. We verify postmortem tissue viability by in situ co-labeling with calcein AM and propidium iodide (PI). The tissue is then imaged by TPEF, followed by manual quantitative analysis of live cellularity.7 Viable postmortem TM has predominantly calcein-positive (live), PI-negative (not dead) cells, while non-viable tissue has a preponderance of PI-positive (dead), calcein-negative (not alive) cells.9

Calcein AM is a well-established cytosolic and viability dye11 that is commonly used in live/dead cytotoxicity kits. The dye is derived from fluorescein diacetate,12 which is converted by esterase activity to a hydrophilic fluorescent dye that is retained in the cytosol. This allows live cells to be identified. Dead cells are not expected to convert calcein AM to its fluorescent form or retain it in the cytosol. Use of calcein AM in the eye has been validated in corneal13 and TM cells.9,14-17 PI is a nucleic acid dye that is membrane impermeable and excluded from viable cells but labels the nuclei of dead cells. PI has been used in flow cytometry since 1992,18 and in cell culture19 and tissue explants.20

The TM in recently postmortem eyes is retained in its original 3D configuration, mimicking the TM in specimens removed during trabeculectomy, a surgical procedure performed in live patients with glaucoma. During trabeculectomy, part of the TM is surgically excised to create an alternative drainage channel for aqueous humor. We seek to analyze this surgically excised viable tissue to explore cellular and molecular diagnostic possibilities for clinical glaucoma. Herein we describe an approach to semi-automate quantification of live and dead cells in TM tissue co-labeled with intravital dyes and imaged by TPEF optical sectioning to improve efficiency of testing. Prior to testing live patients’ tissues, we have sought to simulate analysis in recently postmortem but viable human TM. Here, cellularity was analyzed in 3-dimensional (3D) space based on image reconstructions of tissue optical sections. Isosurface modeling was used to subtract non-specific labeling to make cell counting more specific. We then compared results of our semi-automated counts with manual counts in the same eyes.

Results

Calcein-positive cells (Fig. 1A, arrows) did not generally co-label with PI (Fig. 1B, arrows; Fig. 1C), facilitating specific manual (Fig. 1D) and semi-automated cell counting. Only cells exclusively labeled with PI were considered to be dead.

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Submitted: 10/01/2013; Revised: 11/22/2013; Accepted: 12/02/2013
Citation: Gonzalez J, Tan J. Semi-automated vitality analysis of human trabecular meshwork. IntraVital 2013; 2:e27390; http://dx.doi.org/10.4161/intv.27390
Specific background labeling or particulate debris. This was true provided the best discrimination between labeled cells and non-

may non-specifically label live cells.21 We thus considered calcein-

dead cells is not expected, but PI in high enough concentrations

more dead cells than truly present (μ). Occasionally, calcein-labeled (green) cells also carried signal in

the correct threshold levels; and to best match “Spots” to the

of the reconstruction z-stack data was essential to determining

Fig. 3D labeled cytosols (μ). Manual quantitative analysis

On average it took over 30 min per z-stack to count calcein

and PI-labeled cells one-by-one (Fig. 1D) in serial optical slices. Counting took even longer in 3D image reconstructions, during

which great care was needed to avoid double-counting cells or missing cells in 3D space. We found that cell counting at 8

μm-spaced intervals on the z-axis in 3D image reconstructions best minimized double-counting cells and missing cells during the counting (Fig. 2).

Manual counting of intravital co-labeled tissue wedges from 13 unique donors (A–M) yielded a mean (± SD) of 314 ± 131 calcein-positive cells and 134 ± 78 PI-positive cells, for a total cell count (calcein-positive plus PI-positive cells) of 447 ± 176. The mean percentage of live cells (calcein-positive) to total cells was 70.2 ± 12.8% (Table 1).

Semi-Automated quantitative analysis

Labeled cells were counted with the Imaris Spot creation tool after generating tissue 3D reconstructions from serial optical slices (Fig. 3A–C). 12μm diameter Spot objects (spheres) provided the best discrimination between labeled cells and non-specific background labeling or particulate debris. This was true for cells with PI-labeled nuclei (Fig. 3G) and cells with calcein-labeled cytosols (Fig. 3D). 3D manipulation or reorientation of the reconstruction z-stack data was essential to determining the correct threshold levels; and to best match “Spots” to the fluorescence reconstructions.

Occasionally, calcein-labeled (green) cells also carried signal in the PI channel (red). This likely was due to bleed-through of high intensity calcein fluorescence (green) into the PI (red) channel or occasionally non-specific PI labeling, giving the impression of more dead cells than truly present (Fig. 4). Calcein labeling of dead cells is not expected, but PI in high enough concentrations may non-specifically label live cells.21 We thus considered calcein-positive cells that also had signal in the red channel to have false-positive PI labeling (Fig. 3B; typically low intensity and dark red, compared with higher intensity, bright red true PI labeling of dead cells; eg., Fig. 3A and B) and thus to be alive. To account for the apparent double label signal in some cells (due to calcein bleed-through or non-specific PI labeling), isosurface polygonal models were generated to map all calcein-labeled cells in the green channel (Fig. 3E), as described in “Materials and Methods.” This created a mask for calcein-labeled cells (including calcein-positive cells with red channel signal), allowing their subtraction to leave only exclusively PI-labeled (true dead) cells. This action revealed a new distribution of cells with solely PI labeling (Fig. 3F), from which was generated a revised Spot population (Fig. 3G; red spheres). Imaris then automatically and separately counted red and green Spots. Number of green Spots (for calcein-labeled live cells) relative to total Spots (red spots (exclusively PI-labeled dead cells) plus green spots; Fig. 3H) provided a quantitative assay for tissue viability.

Semi-automated Spot counting of labeled tissue wedges from the same 13 unique donors used for manual counting (A–M) yielded a mean (± SD) of 316 ± 111 calcein-positive cells and 128 ± 70.7 PI-positive cells, for a total cell count of 444 ± 147. The mean percentage of live cells (calcein-positive) to total cells was 71.8 ± 12.2% (Table 1). Semi-automated and manual cell counts were not significantly different with p-values of 0.51, 0.66, and 0.92 for PI-positive, calcein-positive, and total cell counts respectively. Percent live cells for manual (71.2%) and semi-automated (70.2%) methods were close (1.6% difference), with a mean coefficient of variation (SD/mean) for individual tissues of 10.3 ± 7.3%.

Discussion

Primary open angle glaucoma is associated with reduced cellularity in the TM. We have developed a semi-automated quantitative method for high-throughput live/dead cellularity analysis that will assist in cell and molecular testing of viable TM specimens obtained surgically from live glaucoma patients. We have applied the method to recently postmortem but viable human TM that closely simulates TM removed at trabeculectomy. Findings of semi-automated and manual cell counts for live, dead and total cells in 13 unique donors were statistically similar (all \( P > 0.05 \)). Mean coefficient of variation (10%) for percent live
Figure 2. Three-dimensional (3D) reconstruction of human TM labeled with calcein and PI. (A) Isometric view of the corneoscleral meshwork region facing the uveal side. White lines represent frames selected for cropping in the z-axis. Lower-case letters correspond to panels associated with iterative, 8 μm cropped reconstructions. Line “b” bisects a calcein-positive cell, but line “c,” located 8 μm deeper, does not intersect the cell. Lines “e,” “f,” and “g” illustrate that most cells are around/less than 8 μm thick in the z-axis. B-D: Isometric views of optical sections at depths of 16 μm (B), 24 μm (C), and 32 μm (D) from the uveal side; E-G: corresponding optical sections (2D frame) at z-axis depths of 16 μm (E), 24 μm (F), and 32 μm (G). An arrowhead points to a calcein-labeled cell that is visible at 24 μm (B and E) and barely seen at 16 μm (C and F). The brighter signal corresponds to perinuclear concentration of the cytosolic dye. In our semi-automated counting, intensity thresholding parameters are introduced to avoid possible double-counting caused by the dim signal indicated in E. Bar = 10 μm.

Figure 3. Step-by-step illustration of semi-automated cell counting in three-dimensional (3D) reconstructions. (A) 3D reconstruction of calcein (green) and PI (red) co-labeling. (B) PI-labeling in red channel (bright red). Occasionally, false-positive signal (dark red; arrows) is seen in calcein-positive cells due to bleed through from green channel. (C) Calcein-labeling alone in green channel. (D) Spot modeling of calcein-positive cells generated by Imaris (light green spheres). (E) PI-labeled nuclei (red) among calcein-labeled cells represented by “isosurface” modeling (green). (F) Result of using the calcein isosurface (from E) to mask and subtract red signal previously colocalizing with positive calcein-labeled cells (arrows). (G) Spot modeling of exclusively PI-labeled cells (light red spheres). (H) Merge of calcein-positive (26) and exclusively PI-positive (9) spots. Arrows: non-specific red channel signal colocalizing with calcein-positive cells. Bar = 10 μm.
cellularity in individual tissues analyzed by semi-automated and manual cell counts indicated good agreement between the counting methods. Importantly, the estimated time to generate cell counts per specimen was reduced from over 30 min in manual counting to about 3 min in semi-automated cell counting.

Calcein, an intravital cytosolic viability dye, is not known to be toxic to humans and may even be better tolerated than sodium fluorescein in living patients.22 Our methodology employs propidium iodide (PI) as a nuclear counter stain to identify dead cells. This allowed calculation of live cellularity as a percentage of total cells. Nuclear dyes such as PI, ethidium homodimer, DAPI, and Hoechst 33342 are known mutagens23,24 and cannot be used in living patients. For application in live humans, parameters are needed to assess viability based on non-toxic dyes such as calcein AM alone, and would be subject to regulatory approval such as by the FDA. Until such approval, our proposal to analyze surgically-removed viable human tissue provides a suitable alternative to labeling and directly imaging the eyes of live patients.

We took advantage of 3D isosurface modeling to account for non-specific red signal appearing in calcein-positive cells due to calcein fluorescence (green) bleed-through into the PI channel (red) or PI false positive labeling in live cells. Calcein AM is available as different dyes with wavelengths ranging from blue to red. We chose green fluorescence for its bright signal that far exceeds background autofluorescence. This high intensity increased the likelihood of signal bleed-through into the red channel, however. Additionally, PI at high concentrations may non-specifically label live cells.21 As calcein is not expected to label dead cells, we counted calcein-positive cells as alive whether or not signal in the red channel was present. To assist this, isosurface mapping of calcein fluorescence was used to create a mask and subtract calcein-labeled cells (including those also carrying red signal) to leave a pure population of (dead) cells exclusively labeled with PI.

Intravitral co-labeling to assay viability has been reported,11-17,25-29 but semi-automated 3D quantitative analysis of live cellularity is novel. Our semi-automated and manual counts in recently postmortem but viable TM5-8 mimicking TM removed at trabeculectomy yielded very similar results. Cell counting by the semi-automated approach was more efficient, however, taking an order of magnitude less time than manual analysis. The method we have described may also be applied to any tissue, including epidermis, kidneys, lungs, and brain,30-35 that can be imaged by TPEF optical sectioning whether ex vivo or in vivo. Having tested and validated the analytical method in tissue simulating the TM of live patients provides us a rational basis to seek institutional approval to test tissues obtained at trabeculectomy.

### Materials and Methods

**Human donor tissue**

Surgeons of the Doheny corneal service provided residual human donor corneoscleral rim tissue after corneal grafting. Corneal grafting typically occurred within 6 d postmortem.7 In addition, we obtained fresh postmortem eyes within 48 h 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 and Lomb), and

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### Table 1. Live and dead cell counts by manual and semi-automated methods

| Donor | Manual Count | Semi-Automated Count | Percent Live |
|-------|--------------|----------------------|--------------|
|       | PI | Calcein | Total Cells | Percent Live | PI | Calcein | Total Cells | Percent Live |
| A     | 171 | 184   | 355        | 51.8%       | 121 | 184   | 305        | 60.3%       |
| B     | 52  | 220   | 272        | 80.9%       | 100 | 250   | 350        | 71.4%       |
| C     | 71  | 315   | 386        | 81.6%       | 142 | 271   | 413        | 65.6%       |
| D     | 132 | 160   | 292        | 54.8%       | 156 | 203   | 359        | 56.5%       |
| E     | 71  | 363   | 434        | 83.6%       | 95  | 291   | 386        | 75.4%       |
| F     | 37  | 410   | 447        | 91.7%       | 41  | 282   | 323        | 87.3%       |
| G     | 144 | 451   | 595        | 75.8%       | 233 | 313   | 546        | 57.3%       |
| H     | 67  | 206   | 273        | 75.7%       | 24  | 345   | 369        | 93.5%       |
| I     | 126 | 316   | 442        | 71.5%       | 136 | 560   | 696        | 80.5%       |
| J     | 138 | 200   | 338        | 59.2%       | 100 | 277   | 377        | 73.5%       |
| K     | 304 | 395   | 699        | 56.5%       | 234 | 316   | 550        | 57.5%       |
| L     | 173 | 244   | 417        | 58.5%       | 51  | 285   | 336        | 84.8%       |
| M     | 249 | 616   | 865        | 71.2%       | 226 | 531   | 757        | 70.1%       |
| Mean  | 133 | 314   | 447        | 70.2%       | 128 | 316   | 444        | 71.8%       |
| SD    | 78.4 | 131.4 | 175.6     | 12.8%       | 70.7  | 110.8 | 147.0     | 12.2%       |

Unique human donors were designated A–M (n = 13). Percent live cells for manual (70.2%) and semi-automated (71.8%) methods were close (1.6% difference; the mean absolute difference was 10.5%), with a mean coefficient of variation (SD/mean) for individual tissues of 10.3 ± 7.3%.
processed immediately after receipt. Surgical specimens will be transported, maintained and handled in the same manner and conditions. In donor tissue used for graft surgery, 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 and Lomb) on a glass dish and segmented into 8 to 12 wedges. Surgical specimens will not need to be dissected any further which will help retain its natural context.

Intravitral dyes
Hoechst 33342 (nuclear 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.18,19 Hoechst 33342 was used at 1 μg/mL in Dulbecco’s PBS without calcium or magnesium (PBS; Mediatech) and occasionally in combination with other dyes, for 15 min at room temperature (RT) or at 37 °C and 8% CO2. Calcein AM crosses cell membranes and becomes fluorescent in the presence of esterases.30-35 Further enzymatic action by glutathione-S-transferase renders the molecule hydrophilic and unable to exit cells, thus providing a live cell cytosolic label.

The viability co-labeling cocktail was prepared with the following steps: (1) Calcein AM was allowed to come to room temperature; (2) 50 μg Calcein AM was dissolved in 25 μl of DMSO (Cellgro) to generate a 2 mM stock; (3) 2 mM Calcein AM/DMSO stock 1:6666 was diluted in PBS to generate a final Calcein AM concentration of 0.3 μM; and (4) PI was added to the Calcein AM/DMSO/PBS solution to a concentration of 1 μg/mL.

Viability co-labeling with calcein AM and PI
Upon receipt, donor tissues were cut into segments as described above. Some segments were selected to be dead tissue controls and incubated with 0.2% nonionic surfactant (Triton X-100 [TX-100]; EMD Millipore) in PBS (pH 7.4) for 30 min at room temperature. The remaining sections were left in PBS at room temperature during the incubation. TX-100-treated and untreated wedges were incubated with 0.3 μM calcein AM13,27,28,36 and 1 μg/mL PI18,19,37 and/or 1 μg/mL Hoechst 33342 for 30 min at 37 °C and 8% CO2. TPEF was performed immediately after incubation. TX-100 treatment resulted in no identifiable calcein-positive labeling (Fig. 4, n = 6).

We have recently described fluorescence labeling of human TM cells in situ using calcein AM and PI.9 Prior studies have reported effective calcein AM concentrations in TM cells in vitro ranging from 0.15 μM to 4 μM.14,17 We found that a concentration of 0.3 μM was optimal for TM cells in the human donor tissue. Calcein-positive cytosolic labeling was much brighter than the autofluorescence of the structural matrix elements.

TPEF setup
A confocal microscope system (Leica TCS SP5 AOBS MP; Leica Microsystems) coupled to a multiphoton laser (Chameleon Ultra-II; Coherent) was used for TPEF. 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] to a nondescanned photomultiplier tube detector (Hamamatsu). Images were collected as z-stacks (xyz, 600 Hz, bidirectional) using 512 x 512 or 1024 x 1024 pixel resolution and 16 x 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

Figure 4. Examples of calcein (green) and PI (red) co-labeling in detergent-killed tissue (A) and in tissue exhibiting false-positive red signal due to calcein bleed-through from green channel (C). (A) Triton X-100-treated tissue with dead cells. This revealed extensive PI-labeled nuclei (arrows; dead cells) but no green cytosolic labeling (no live cells). Collagen- and elastin-derived autofluorescence7,10 is seen (green channel; asterisks). (B) Green fluorescence channel only. In tissue without detergent, image settings to accomodate bright green fluorescence from calcein-positive cells (arrowhead) render autofluorescence too dim to see. (C) Red fluorescence channel only. A single PI-positive cell is seen (arrow; bright red). The same calcein-positive cell from B is also seen in the red channel (arrowhead; dimmer red). Bar = 10 μm.
by argon laser excitation at 488 nm and emission detection at 500 to 535 nm using an internal PMT detector (Hamamatsu). These imaging settings allowed AF of fine structures of the TM such as ECM beams, plates, and fibers to be seen.10 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]); cropped, resized, and fit into figures using a graphics editing program (Photoshop CS5; Adobe). Isosurface mapping, polygonal reconstruction and 3D manipulation were performed in Imaris (Bitplane).

**Manual cell counting**

Live and dead cells were counted through entire z-stacks using 8-μm steps through the z-plane (Fig. 2). For example, if a z-stack comprised optical sections at 1-μm intervals, only cells from every eighth frame were counted. This minimized the chance of double-counting cells. Depending on the concentration of PI used, PI occasionally labeled live cells, albeit with lower fluorescence intensity compared with dead nuclei. To obviate this, PI concentration was titrated during assays, going as low as 1 μg/mL. Calcein labeled cells (green; cytosol-labeled) were considered to be alive. Exclusively PI-positive cells (red; nuclear-labeled) were considered dead. Occasionally, calcein-labeled cells also bore red signal, either from calcein bleed-through (Fig. 4B and C) or from PI non-specific labeling; these cells were considered to be alive as calcein is not expected to label dead cells. Treatment of tissue with TX-100 (to kill cells; EMD Millipore) showed universally positive PI labeling but negative calcein labeling (Fig. 4A). This represented our dead controls. Percentage live cellularity was determined by calculating the ratio of calcein-positive cells to the total number of cells (sum of calcein-positive [live] cells and PI-positive [dead] cells).

**Semi-Automated cell counting**

Entire image stacks were loaded into Imaris 7.3.0. The stack was viewed in “Surpass” mode. The volume mode was set to “Blend.” Gamma was set to 1.00 and Opacity to 24.01% for both channels. “Min” and “Max” levels were adjusted for each channel to approximate the fluorescence data. A generally good starting point was a minimum of 5480 and maximum of 21690. Next, “Background Subtraction” was used with a “Quality” filter with a “Threshold” adjusted manually to best approximate the fluorescence data. These settings were applied to the entire data set and the software automatically determined the total number of spots.

**Volumetric deletion of bleed-through signal**

Occasionally, red signal was seen in conjunction with calcein labeling (green) in the same cell. We considered this appearance to represent calcein bleed-through (from the green channel; see Fig. 4B and C) or non-specific PI labeling of live cells. To count exclusively PI-labeled cells (e.g., Fig. 4A), calcein-labeled cells (with or without red signal) were subtracted by setting their pixel intensity values to zero. To perform this adjustment, a polygonal mesh approximation of calcein fluorescence data in 3D reconstructions was generated in Imaris. This “Isosurface” was created using “Surfaces” and “Surpass” modes for the calcein channel in the software. Smooth surface mapping was used that at least matched X-Y resolution. Thresholding for absolute intensity was adjusted manually to best approximate the fluorescence data. A generally good starting point was a minimum of 10025. A filter for number of voxels with minimum value set manually to 10.0 was used. Application of these settings to the entire calcein data set created a new “Surfaces” object, which was used as a mask with voxel value set to 0.000. A mask layer was also created for signal in the red channel that shared 3-D localization with calcein-positive voxels.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

Special thanks to doctors of the Doheny Eye Institute Corneal Service for providing human corneoscleral donor rim tissue (Drs Martin Heur, Hugo Hsu, Neda Shamie, Olivia Lee, Bibiana Jin Reiser); Drs Jim Burford and Janos Peti-Peterdi (USC Multiphoton Core); and Ernesto Barron and Dr David Hinton (Doheny Vision Research Imaging Core) for sharing imaging expertise.

**Grant Support**

National Institutes of Health, Bethesda, MD, Grants EYO20863 (JCHT), EYO3040 (Doheny Vision Research Institute Imaging Core), 1S10RR24754 (USC Multiphoton Core), and a Kirchgeisser Foundation Research Grant (Tan JCH), American Glaucoma Society Mentoring for Physician Scientists Award and Young Clinician Scientist Award (Tan JCH), Career Development Award from Research to Prevent Blindness (Tan JCH), and an unrestricted grant from the Research to Prevent Blindness, Inc., New York, NY.

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