The Structural Relationship between the Microvasculature, Neurons, and Glia in the Human Retina


PURPOSE. To develop a new technique for detailed study of the spatial distribution of retinal and choroidal microvasculature and their relationship to neurons and glial cells at the cellular level in human cadaveric eyes.

METHODS. Twenty-six human donor eyes were used. Wherever possible, the central retinal artery and a branch of the posterior ciliary artery were individually cannulated and perfused with oxygenated Ringer’s solution with 0.5% bovine serum albumin. The perfusion pressure was continuously monitored. Once residual blood was washed out, the perfusate solutions were switched to fixative, membrane-permeabilizing solution and selected labeling solutions. The eyes were then immersion fixed and the retina and choroid flat-mounted for immunolabeling and confocal imaging before cryosectioning. The microstructures of vascular, glial, and neuronal cells in the retina and the stroma in the choroid were studied.

RESULTS. The retinal microvasculature was fully perfused and stained by cannulation of the central retinal artery. Regional distribution of choroidal vasculature perfusion was dependent on the specific feeder artery cannulated. The detailed spatial relationship between endothelial cells, glial cells, and neurons at the cellular and subcellular levels was identified with confocal microscopy and immunohistochemical labeling of retinal sections. In the choroid, endothelial cells were clearly identifiable down to the level of the intracellular cytoarchitecture of the choriocapillaris, along with their relationship to Bruch’s membrane and the feeding and drainage vessels.

CONCLUSIONS. A microperfusion fixation and staining technique has been developed that allows studies of the structural relationships of vascular, glial, and neuronal elements at the cellular level in human donor eyes. (Invest Ophthalmol Vis Sci. 2010;51:447–458) DOI:10.1167/iovs.09-3978

Although it is only 2% of total body weight, the brain uses 20% of the total energy consumed by the body.1 The energy demand of the retina on a per gram basis has been described as higher than that of the brain.2–6 Well-regulated blood flow within the brain and retina is thus vital to maintain energy-dependent processes and to clear metabolic by-products produced by neuronal activity. It has been demonstrated that even relatively small reductions in blood flow can have deleterious effects.7 The human retina is vulnerable to a wide range of retinal diseases with a vascular component, and angiogenic ocular conditions represent the leading cause of irreversible vision loss in developed countries.8 Such diseases include diabetic retinopathy, vascular occlusion, and age-related macular degeneration.9,10 The vulnerability of the retina presumably stems in part from the need to limit the extent of retinal vasculature to allow a clear light path to the photoreceptors. The outer retinal layers are completely avascular and are dependent on metabolic support via diffusion from the retinal and choroidal vascular beds.11 In the foveal avascular zone the human retina is presumably almost totally dependent on choroidal support alone, as demonstrated for oxygen delivery to the monkey fovea.11 The retinal and choroidal vascular beds have remarkably different properties. The retinal circulation is relatively sparse, with a well-developed autoregulatory capacity that must be locally controlled as there is no autonomic innervation.12,13 The choroid is highly vascularized and has extensive autonomic innervations but is often reported to have little regulatory ability to respond to changes in systemic oxygen levels.14 Metabolic and vascular regulation in the retina is presumably dependent on the interaction between many cellular elements including the endothelium, smooth muscle cells, glia, and neurons. The microvasculature is a complex and interconnected network under tight regulatory control that exists in intimate communication with neurons and glia. To investigate the undoubted complexity of such relationships requires careful consideration of the spatial and functional characteristics of these different cell types. In this regard, it would be valuable to have a better understanding of the three-dimensional (3-D) structure of vascular, glial, and neuronal elements at the cellular level.

The vascular endothelium not only serves as a dynamic, semiselective barrier that regulates transport of fluid and macromolecules between blood and the interstitium, it also synthesizes and releases various factors that modulate angiogenesis, inflammatory responses, homeostasis, and vascular tone.15,16 Therefore, to better understand the regulation of endothelial cell (EC) barrier function, the molecular, and functional analyses of protein kinases and phosphatases in ECs are equally important. Numerous recent findings pertaining to ECs support their complexity both in structure and regulation.

Although studies in animal models can provide much useful data, understanding these relationships in the human retina is fundamental for identifying the key factors in ischemic retinal diseases. Of particular relevance is the specialized macula region. The present study seeks to develop new techniques for...
the microperfusion of human donor eyes and the use of established immunohistologic and dye labeling techniques to further explore the spatial distribution of vascular, neuronal, and glial cells in the human retina and choroid. With such techniques, we demonstrate that it is possible to label endothelial cell structures, astrocytes, neurons, and their intracellular structure using available markers in the same preparation having preserved the structural relationship between these elements. In contrast, with more commonly used techniques such as vascular casting, trypsin digestion, and histochemistry, much of the structural relationship between vascular, glial, and neuronal elements is lost. A significant advantage of our new technique is the capability of integrating the detailed cellular and subcellular information about the ocular vasculature and neurons, glia, or stroma from both flatmounted and sectioned specimens using multiple markers. This integration can effectively avoid misinterpretation obtained from single sections or flat-mounted preparations using single markers. Establishing normal baseline data in healthy eyes will form the basis of future work in eyes with a known history of ocular disease where disruption of the relationship between these elements may prove to be a factor in disease pathogenesis.

**Materials and Methods**

This study was approved by the human research ethics committee at the University of Western Australia. All human tissue was handled according to the tenets of the Declaration of Helsinki.

**Human Donor Eyes**

A total of 26 human eyes from 18 donors were used for this study. All eyes were obtained from the Lions Eye Bank of Western Australia or Donate West, the West Australian agency for organ donation. We received the eye bank eyes after removal of corneal buttons for transplantation. None of the eyes used in the present study had a known history of eye disease. The demographic data, cause of death, and postmortem time to eye perfusion or fixation for each donor are presented in Table 1.

**Preparations**

The posterior globes of donated eyes were delivered to our laboratory on ice and were either used immediately or kept in ice-cold oxygenated Ringer’s solution until use. The feasibility of successfully cannulating the central retinal artery is very dependent on optic nerve length. Eyes with sufficiently long optic nerves were selected for perfusion studies using both the central retinal artery and a branch of the posterior ciliary artery. Eyes with short optic nerves were either used for posterior ciliary artery perfusion alone or immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer solution for whole-eyecount immunolabeling. Postmortem time to initial perfusion or immersion fixation ranged from 5 to 20.5 hours.

**Perfusion Technique for Fixing and Labeling the Vascular Endothelium of Retinal and Choroidal Microvasculature**

A schematic of the perfusion system is shown in Figure 1 along with a photographic inset of a perfused human eye (Fig. 1A2). Cannulas are seen in the central retinal artery (CRA) and in a branch of the posterior ciliary artery. Two double suture ties (8/0) were used to secure each cannula. Syringe pumps (model 22; Harvard Apparatus, South Natick, MA) were used to deliver an adjustable flow of perfusate (typically 50 μl/min) and the perfusion pressure was continuously monitored through conventional transducers (Cobe, Arvada, CO), each connected to a bridge amplifier (model 5B38-02; Analog Devices, Norwood, MA) and recorded on a chart recorder (LR8100; Yokogawa, Tokyo, Japan). The flow rate was chosen to ensure that intravascular pressures did not exceed the physiological range. Direct measurements of intravascular pressure in canine eyes indicated that the pressure in the retinal artery is approximately 70% of that in the aorta.\(^\text{17}\) Assuming a similar relationship holds in humans, then a mean systemic blood pressure of 100 mm Hg would produce an average intravascular pressure in the retinal artery of ~70 mm Hg. At a normal IOP of 15 mm Hg, the mean transmural pressure in the retinal artery would therefore be ~55 mm Hg. In our donor eyes, with the cornea removed, the IOP is essentially 0. Thus the transmural pressure is equal to the intravascular pressure. We chose a baseline flow rate (50 μl/min) that resulted in average transmural pressures in the central retinal artery close to 50 mm Hg.

The standard perfusate solution for washout was oxygenated Ringer’s solution with 0.5% bovine serum albumin. At least 15 minutes was allowed to flush out any residual blood. Then, the following solutions were perfused through in order: 4% paraformaldehyde in 0.1 M phosphate buffer solution (20 minutes), 0.1% Triton-X 100 in 0.1 M phosphate buffer solution (5 minutes), 0.1 M phosphate buffer solution (20 minutes), dye (30 μl Alexa Fluor 546 or 635 phalloidin and Hoechst 1.2 μg/ml in 0.1 M phosphate buffer [120 minutes]; A22828 or A34054 Invitrogen, Carlsbad, CA; and bis-benzimide H 33258, cat no. B2261; Sigma-Aldrich, St. Louis, MO), and 0.1 M phosphate buffer (30 minutes). Some eyes were perfused with TRITC-lectins (50 μg/750 μl) in

### Table 1. Donor Eye Details

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<tr>
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After thawing to room temperature, the retina was floated in a 0.1 M cryoprotected in 30% sucrose. It was then stored at sodium borohydride (tetrahydroborate) in phosphate buffer overnight, in 0.1 M phosphate buffer on a shaker for 1 to 2 days and floated in 1% various retinal cell components. In brief, the whole retina was washed col by Xiao and Hendrickson,18 to study the relationship between tem may be immunolabeled as a wholemount according to the proto-

Wholemount Retina Immunohistochemistry

Retinas that have been perfusion-stained or immersion postmor-
tem may be immunolabeled as a wholemount according to the proto-
col by Xiao and Hendrickson,18 to study the relationship between various retinal cell components. In brief, the whole retina was washed in 0.1 M phosphate buffer on a shaker for 1 to 2 days and floated in 1% sodium borohydride (tetrahydroborate) in phosphate buffer overnight, cryoprotected in 30% sucrose. It was then stored at −80°C until used.

Intravascular VE-cadherin Immunohistochemistry

We used a light microscope (E800; Nikon, Tokyo, Japan) equipped with Plan Apo lenses ranging from ×4, ×10, ×20, ×40 (oil), ×60 (oil), to ×100 (oil). In addition to the usual broadband UV blue and green filter blocks, a special triple filter block was used for simultaneous imaging of DAPI (or Hoechst)/Texas red/FITC. ACT-1 software allowed imaging at nine levels of resolution, control of exposure time, and labeling of image files. Images were captured with a digital camera (DXM1200; Nikon).

Confocal Imaging

A confocal imaging system (C1; Nikon) equipped with three lasers (405, 488, and 532 nm) was used in conjunction with a fluorescence microscope (E800; Nikon). Imaging was performed with the system software (EZ-C1, ver. 3.20, Nikon). Confocal imaging was performed simultaneously for the different wavelengths with emission signals separated into different channels. Imaging began at low magnification. Specific regions were zoomed in with high-power objective lenses (×40, ×60 plan apochromatic oil lenses) for detailed imaging. A Z-series was taken through to a depth of 100 μm, to obtain 3-D information on retinal microvascular architecture and to study labeled structures in the vascular endothelium and the retina. The three wavelengths enabled analysis of the triple labeling of the vascular endothelium and retina.

Region Identification and Immunohistochemistry

After imaging, specific regions (e.g., macula, perifovea, and surround-
ing retina) could be dissected out and embedded in OCT for cryosec-
tioning. Frozen semi-serial sections were mounted on gold slides (Eire Superfrost PLUS, ERIFT-9811GLPLUS-006E; Biolab, Decatur, GA) and allowed to air dry for an hour before staining. Sections from the same
region were labeled with antibodies for identification of different types of retinal neural and glial cells (Table 2). Intensity of staining with particular markers can increase or decrease when tissues are exposed to certain physiological or pathologic conditions. Caution is therefore needed to avoid coming to false conclusions on the basis of single markers.

**Choroid Preparation**

After the retina was detached from the posterior globe as far as possible, the RPE layer was carefully removed with a ferret hair brush and the RPE away washed from the choroid with buffer. A cut was made along the inferior meridian of the intact choroid/sclera eye cup for the purpose of orientation before detachment of the choroid from the sclera. The choroid was peeled from the underlying sclera with the help of the brush. The area around the macula may require the help of a metal ‘scoop’ or cutting with fine scissors. The dissected choroid was then flat mounted in glycerol, retina side face up, and multiple radial cuts were made to help flatten the specimen before coverslipping and imaging.

**Statistical Analysis**

Kolmogorov-Smirnov testing was performed on all data before analysis to determine whether the data were normally distributed. Normally distributed data were analyzed with ANOVA with post hoc factor comparison performed using a paired Student’s t-test with Bonferroni correction. Non-normally distributed data were analyzed using ANOVA on ranks with the Tukey test used for post hoc paired analysis. All correction. Non-normally distributed data were analyzed using ANOVA.

**RESULTS**

**Perfusion and Staining Technique**

Our results demonstrated that when the central retinal artery was cannulated and perfused the retinal microvasculature was well stained, and the endothelial cells and nuclei of the retinal vasculature were clearly labeled. Choroidal vasculature perfusion staining was highly dependent on the absence of excessive shunt flow through collateral arterial branches. Vascular leakage in the retinal circulation was not observed when perfusion pressure was kept within the physiological range. Figure 1 shows an example of how perfusate pressure varies at different stages during a retinal perfusion. Pressure quickly stabilized soon after perfusion with Ringer’s solution with 0.5% bovine serum albumin commenced. Pressure then tended to drop slightly during further washout. Averaged perfusion pressure in the central retinal artery cannula from 20 eyes after 15 minutes of initial perfusion was 47.9 ± 5.0 mm Hg. The vascular resistance to perfuse flow can be determined by the ratio of perfusion pressure to perfusate flow (50 μL/min). The estimated resistance of the human retinal vasculature after the initial flush was therefore ~1 mm Hg/μL/min (range, 0.9–1.3 mm Hg/μL/min). After a switch to 4% paraformaldehyde in 0.1 M phosphate buffer, the average perfusate pressure increased significantly to 65.4 ± 6.0 mm Hg (P < 0.001) at 15 minutes. Triton-X 100 in 0.1 M phosphate buffer solution perfusion (5 minutes) produced an average perfusate pressure of 58.0 ± 5.5 mm Hg. Flushing with 0.1 M phosphate buffer solution (15 minutes) reduced the average perfusate pressure to 42.9 ± 4.0 mm Hg, which was not significantly different from the pressure at the end of the initial flush. The perfusion pressure of the choroidal vasculature was much lower than that for the retinal vasculature at the same flow rate. There were remarkable anatomic variations in LPCA/SPCA distribution and their branches as previously reported.27,28 SPCAs do not have the characteristics of end arteries, and they anastomose freely with each other. In addition, there is a segmental distribution of perfusate flow and multiple feeding and drainage patterns within the choroidal vasculature. When perfusing selected regions of the choroid, careful selection from available feeder arteries is needed before cannulation. In our study, in some cases, more than one cannulation or ligation of branches was required. The perfusate pressure range during choroidal perfusion varied greatly between eyes, with unknown shunt flows precluding a sensible estimate of choroidal vascular resistance.

**Distribution of Retinal Microvasculature**

Fluorescence and confocal imaging were performed on the perfused and stained specimens of the retina and choroid. Fluorescence imaging allowed for quick scanning to identify regions of interest and speedily capturing of images. Confocal imaging sampled from specified region provided spatial distribution, 2-D or 3-D, and at the cellular level with high magnification. Figure 2 shows confocal images of the superior–temporal quadrant of a perfusion stained retina from the left eye of a 43-year-old man (donor F). This specimen was immunolabeled for VE-cadherin by intravascular perfusion. The low magnification image (Fig. 2A) is a projection of sections over a depth of 120 μm. It shows a well-perfused stained intact retinal microvasculature consisting of arterioles, venules, and capillaries. The retinal capillaries were diffusely distributed. The more magnified image (Fig. 2B) is a color composite of three projected stacks at different Z levels of the marked region. The three stacks were pseudocolored from superficial to deep in the order of red, green, and blue. One can use this technique to measure the capillary meshwork density at different layers.
Although there is a slight slanting of this specimen, the 3-D distribution of the retinal microvasculature was clearly demonstrated. A regular loop of deep capillaries was clearly seen (blue coloring). The interrelationship of the connection and depth between the arteriole, venule, superficial and deep capillaries was able to be defined. There was a capillary-free zone along the arteriole in the superficial capillaries but there were several deep capillaries passing under this arteriole.

Figure 3 shows the confocal images of the macular region of a perfusion-stained retina from a 52-year-old man (donor A). The paired images show the macular region at low magnification (Fig. 3A) and an enlarged image (Fig. 3B) of the fovea labeled for filamentous actin. Depth information has been included for this stack of 106-μm images. The top 50 μm was pseudocolored red and the remainder pseudocolored green. At low magnification (Fig. 3A), the depth coloring appears to indicate a deepening slope of the vasculature to deeper layer toward the temporal region. The alternating arteriole (a) and venous (v) pattern of the macula area is clearly seen. Most small branches of the arterioles that arise from the arterioles in the innermost retinal layers pass directly into the retina, often at right angles to the vessel of origin. They connect with the venule capillaries at all levels, forming a dense capillary plexus suspended like a hammock between the feeding arterioles and draining venules. At least seven pairs of arterioles and venules were found in this macular area and at least three pairs were intimately connected to capillaries bordering the avascular zone (Fig. 3B). Sometimes capillaries were clearly visible going across the fovea in the deeper layer of this macular region. This was seen in 5 of the 18 eyes in which retinal perfusion staining was studied. The presence of a capillary crossing the fovea causes the capillary-free zone to be irregular in shape and size in area. Changing the scanning depth of the confocal microscope indicated that the sharp bend in the foveal capillary was deep within the retina and adjacent to the foveal pit. Figure 4 is an example of confocal images of the macular region of a perfusion-stained retina from a 61-year-old man (donor R). In this eye there was not a capillary crossing the fovea so the capillary-free zone was larger than in the previous example (Fig. 3).

Intracellular Structure of Endothelial Cells of the Retinal Microvasculature

In addition to the 2D and 3D vascular distribution, the intracellular cytoskeleton of the endothelial cells could also be defined. Figure 5 shows confocal images of perfusion-stained retinal vessels labeled for F-actin from the left eye of a 51-year-old man (donor C) and VE-cadherin from the left eye of a 43-year-old man (donor F) showing intracellular structure and nuclei counterstained with Hoechst. The F-actin in both the endothelium and vascular smooth muscle cells were tagged by phalloidin. The shape of endothelium was outlined by cell border located F-actin (peripheral border staining, PBS) in the retinal arteriole, venule, and capillary. However, structured filamentous structure (stress fibers, SF) was found inside the arterial endothelium, whereas a dotted/diffuse appearance of F-actin staining was seen in the endothelial cytoplasm of the vein and capillaries. VE-cadherin staining was seen only in the endothelium, predominately in cell borders. In the arteriole, the pattern of endothelium was spindle shaped and the nucleus was elongated with its long axis parallel to the long axis of the cell. The smooth muscle cells show much greater F-actin staining intensity, lying perpendicular to the long axis of the endothelial cells. In the venule, endothelial cells show a more polygonal shape, with round nuclei and only sparse smooth muscle cells that were irregular in shape and orientation. It was not easy to indentify individual endothelial cells, even when the capillary was clearly stained by both F-actin and VE-cadherin and scattered nuclei could be easily defined.

![Intracellular Structure of Endothelial Cells of the Retinal Microvasculature](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933452/)
The Spatial Relationship between Retinal Neurons, Microvasculature, and Glial Cells

It is important to know the spatial relationship between retinal neurons, microvasculature, and glial cells. Figure 6 shows triple staining of a perfusion-stained retina of a 43-year-old man (donor F). The microvasculature had been perfused and labeled for VE-cadherin, which is endothelium specific. The wholemount retina was subsequently immunolabeled for glial fibrillary acidic protein (GFAP) and the nuclei labeled with Hoechst by immersion immunohistochemistry. GFAP is commonly found in astrocytic processes and in activated Müller cell processes. Figure 6A is a projected image of a stack from the inferior temporal quadrant taken at low magnification. Astrocytes were seen in the superficial
retina forming a honeycomb pattern. The retinal microvasculature, including an arteriole and venule and the capillary network, can also be seen. Not all astrocytes were associated with retinal vasculature. The orientation of the astrocyte processes related to microvessels also varied. Some processes ran along microvessels, but some ran obliquely or almost perpendicularly. Staining intensity also was not even. Under higher magnification, a starlike shape of astrocyte label can be seen (Fig. 6B) with several profuse and radially oriented branch processes as described previously.29 The filamentous distribution of GFAP staining can be seen within the cell soma. The nuclei of the astrocyte were not clearly seen in this projected image of a stack; however, we were able to identify them in individual optical sections (Fig. 6B; inset). The astrocytic processes appeared nonuniform in caliber and tended to form bundles. Astrocyte processes wrapping over larger arterioles often showed some finer processes/extensions (Fig. 6B); however, the number of astrocytic process wrappings in larger arterioles appeared much less frequent than in smaller arterioles, venules (Fig. 6C), and capillaries (Fig. 6D). There were some fine processes running between the neuronal nuclei. A detailed relationship between astrocyte, capillaries, and small venules was evident in Figure 6D where a coarse bundle consisting of many processes oriented perpendicularly toward and wrapped a capillary while other processes ran along the capillaries. There were numerous

FIGURE 5. Confocal images of the intracellular structure of retinal vascular endothelial cells: (A) a retinal artery (A), (B) vein (v), and (C) capillaries, with intravascular perfusion staining for F-actin (donor C). Confocal images of (D) retinal artery (A), (E) vein (v), and (F) capillaries, labeled for VE-cadherin (donor F). The nuclei (N, blue) were counterstained with Hoechst. The F-actin labeling is visible inside the retinal endothelium and vascular smooth muscle cells (★). Insets: Cell border F-actin staining outlines the shape (yellow arrowheads) of the endothelium in the retinal artery and vein. F-actin in the endothelial cytoplasm can be seen with a dotted/diffuse appearance in the vein and capillaries, or in a more structured filamentous structure (stress fibers, white arrowheads) inside the arterial endothelium. VE-cadherin staining is endothelium specific and can be seen clearly at the endothelial cell border (yellow arrow, inset) in the retinal artery, vein, and capillaries. Scale bar: (A–C) 20 μm; (D–F) 50 μm.
Immunohistochemistry in Retinal Sections

A map of distribution of the retinal microvasculature and glial cells can be obtained with a retinal flat-mount preparation. Histologic sections from the specific location as provided by the retinal map may be used for immunohistochemical study. Figure 7 shows some examples of immunolabeling in the cryosection. Most labeling can be performed in the cryosection preparation which provides valuable information from retinal cellular layer in addition to flat-mounted preparation which may be limited by the label’s penetration capability and the confocal scanning properties of deeper tissue.

Figure 7 shows confocal images of retinal cryosections from a 66-year-old man (donor E). The sections were taken from the parafoveal region and have been double immunolabeled and counterstained with Hoechst to visualize the nuclei. Negative control sections (Figs. 7G, 7H) processed using the same protocol in the absence of primary antibodies show no nonspecific staining from the secondary antibodies. Paired images (Figs. 7A–F) show double labeling for recoverin and cellular retinaldehyde binding proteins (CRALBP); long- and medium-wavelength opsin and synaptophysin (SYN), and neuroglobin and mitochondria (MITO). The left panel shows images displaying all three labels (Alexa Fluor 546, 488 and Hoechst) and the right panel shows the corresponding sections highlighting only two labels (Alexa Fluor 488 and Hoechst). Recoverin immunolabeling was seen throughout the photoreceptor cell length and weakly picked up by the remainder of the retina. CRALBP labeling was found in the Müller cell end feet at the inner limiting membrane (ILM), with fine fibers extending through the inner plexiform layer, the processes in the region of Henle’s fibers, and in the interphotoreceptor matrix, stopping at the outer border of the photoreceptor nuclei. CRALBP also labeled the retinal pigmented epithelium (RPE; Fig. 7B). Anti-L/M opsin (Fig. 7C) was picked up by a regular array of cones staining most strongly in their outer segments, strongly positive in their outer segments, and through to the outer plexiform layer. Synaptophysin (Fig. 7D) was clearly present throughout the inner plexiform layer and the outer plexiform layer where synapses are located. Neuroglobin (Fig. 7E) appears to be rather widespread, with stronger labeling seen in the Henle’s fibers, region between mitochondria rich inner segment and the photoreceptor nuclei, and the nerve fiber layer/inner limiting membrane. Some cell soma of the inner nuclei layer has also stained positive for neuroglobin. In Figure 7F, mitochondria are clearly labeled in the inner segments of the photoreceptors, outer plexiform layer, inner plexiform layer, the somas of ganglion cells, and the basal portion of the retinal pigmented epithelium.
FIGURE 7. Confocal images of retinal cryosections from a 66-year-old man (donor E). The postmortem time was 20.5 hours before the globe was placed in 4% paraformaldehyde for fixation. The sections were taken from the parafoveal region and have been double immunolabeled and counterstained with Hoechst (blue) to visualize nuclei positions. Paired images show double labeling for recoverin (A) and CRALBP (B). Long- and medium-wavelength opsin (C), synaptophysin (D, SYN), neuroglobin (E), and mitochondria (F, MITO). Left: images displaying all three labels (Alexa Fluor 546, 488, and Hoechst); right: the corresponding sections highlighting only two labels (Alexa Fluor 488 and Hoechst). (A) Recoverin labeling is visible throughout the photoreceptor cell length; it was weakly picked up by the rest of the retina. (B) CRALBP labeled Müller cell end feet at the inner limiting membrane, with fine fibers extending through the inner plexiform layer, strongly labeling the processes in the region of Henle’s fibers in the interphotoreceptor matrix and stopping at the outer border of the photoreceptor nuclei. CRALBP also labeled the RPE. (C) Anti-L/M opsin was picked up by a regular array of cones, staining most strongly positive in their outer segments and strongly present in the inner segments, soma, and their axonal processes through to the outer plexiform layer. (D) SYN is present throughout the inner plexiform layer, and the outer plexiform layer. At higher magnification, plate and disc shapes of the synaptic processes are identifiable. (E) Neuroglobin appears to be rather widespread with stronger labeling seen in the Henle’s fiber region, between the mitochondria-rich inner segment and the photoreceptor nuclei, and in the nerve fiber layer/inner limiting membrane. Some cell soma of inner nuclei layer also stained positive for neuroglobin. (F) Mitochondria were found in the inner segments of photoreceptors, outer plexiform layer, inner plexiform layer, the somas of ganglion cells and the basal portion of the retinal pigmented epithelium. (G, H) Cryosections processed in the same protocol but with the absence of primary antibodies served as the negative control. Confocal images of a negative control section collected simultaneously via three-channel (405, 488, and 532 nm) and two-channel (405 and 488 nm) excitation, indicate the absence of nonspecific staining of the retinal tissue. Autofluorescence is present at the RPE level and in red blood cells within the blood vessels. Counterstaining with Hoechst shows the location of the inner and outer nuclear layers. Scale bar, 100 μm.

Choroidal Microvasculature

The microperfusion labeling technique also provides an opportunity to explore the distribution and cytoarchitecture of the choroidal vasculature. Figure 8 shows some examples of confocal images of human choroidal vasculature. Figure 8A shows a confocal image taken from the nasal region of a 61-year-old (donor I). Perfusion via a SPCA allowed staining for endothelial glycocalyx, microfilament, and nucleic acid dye Hoechst enabled visualization of the choroidal vasculature. This image was taken with a 10X objective lens at the level of the choriocapillaris. The TRITC-conjugated lectins have attached onto the endothelial surface of the choriocapillaries indicating the presence of glycocalyx in the endothelium. A dense meshwork of short, interconnecting capillaries may be seen as well as some larger supplying or draining vessels. The arteriole and venules can be determined by tracking back to the larger vessels in the mid or deeper choroid (image not shown). High-magnification images of choriocapillaries that had been labeled with Alexa Fluor 635 phalloidin and with Hoechst are shown in Figure 8B. The image was taken at the choriocapillary level from the perfusion-stained macular region. A dense interconnecting network consisted of very short, large-caliber capillaries. Each capillary had only a few endothelial cells. The peripheral border staining of these endothelial cells of the choriocapillaries is clearly visible. Most endothelial cells had an irregularly polygonal shape. Microfilament or F-actin staining inside the endothelium of the choriocapillaries is clearly visible in a dispersed, dotted, or fragmented pattern inside the cytoplasm. F-actin peripheral border staining indicating the endothelial junction was found at the surface facing the Bruch’s membrane. Some unevenness was noted along the cell border, suggesting the aggregation of microfilaments at the thickened points. The endothelial nuclei were notably located in one pole of the endothelia, often away from the surface of Bruch’s membrane, or toward the edge of the 2-D capillary wall. Figure 8 (C1, C2, and C3) is derived from a stack of confocal images of the choroidal vasculature from a 45-year-old (donor G). The choroid was perfusion-stained for endothelial cell F-actin and nuclei. The stack was 60 μm in depth with optical sections 0.3 μm apart, taken at high magnification (×40 oil lens). A single optical section shown in Figure 8C1 is almost half way through the optical stack and the crossing point of the x- and y-axes was placed near the base of a choroidal capillary, to examine the feed vessel in relation to the choriocapillary bed (Fig. 8C1, blue arrows). Because the sample was slightly tilted, other areas of the section plane cut through the choriocapillaries themselves. The location of the C1 optical section in the stack is indicated by the white line running lengthwise along the two cross-sections C2 and C3. Cross-sectional views of the whole stack at x- and y-axes are...
shown correspondingly as Figures 8C2 and 8C3. The choroidal surface of the optical stack can be identified in both images. The exact level of the single optical section of 8C1 related with the choroidal surface can be determined by z-level sections of Figures 8C2 and 8C3 (white lines). Such 2- and 3-D geographic maps and cytoarchitecture of the choriocapillaries and the relationship between the choriocapillaries and their supply and drainage vessels, as well as Bruch’s membrane, allow the interrelationship of these structures to be studied in great detail.

**DISCUSSION**

The purpose of this study was to develop a new technique to study human donor eyes to investigate the spatial 2- and 3-D relationships between microvasculature, glial cells and neurons in the retina and between the choriocapillaries, supply, and drainage vessels and Bruch’s membrane at both the geographic distribution and cytoarchitecture level. Our results demonstrate that this technique can effectively wash out blood from the ocular microvasculature, fix the tissue, and deliver dye or antibodies specific to the endothelium, glial cells and neurons. Using confocal and fluorescein microscopy we obtained information from specific regions of interest. The information gained from such studies of human donor eyes has the advantage that the structural relationship between many cell types is preserved. This structural preservation may prove advantageous when compared with the more conventional techniques such as trypsin digestion, vascular casting, or routine histology.

Cannulation of such fine vessels presents a technical challenge. It is also important that any residual blood be washed out. We were careful not to allow perfusate pressure to exceed the normal physiological range, since it is likely that the vasculature would be weakened in donor tissue obtained postmortem, and we wanted to avoid rupture of the vascular wall. In establishing this technique we were aided by our previous experience in a range of isolated eye or isolated vessel preparations. The position of the central retinal artery on the optic nerve was relatively consistent in all donor eyes. Since there is only one feeder vessel and no collateral branches, once cannulated, the entire retinal vasculature can be easily perfused. Our results showed that the endothelial cells of the entire retinal vasculature, including the capillaries, were well stained. However, there were significant anatomic variations in the long and short posterior ciliary arteries in terms of their locations and number of branches. It was often not possible to perfuse the entire choroidal circulation. However, careful selection of which artery to cannulate and the ligation of leakage pathways allowed the region of interest to be adequately perfused.

The human donor eyes selected for this study were obtained from donors with no previous history of ocular disease.
The data obtained therefore is presumably representative of the normal human eye. The present study is limited to a demonstration of the feasibility of such a technique in human donor eyes. Clearly, there is scope for similar studies in a range of human retinal diseases and in detailed studies related to aging processes in the human eye.

In general, our results clearly showed a fully perfused microvasculature in the retina and the region of choroidal circulation perfused as demonstrated in Figures 2 and 8A. High-magnification confocal images of the blood vessels also showed no erythrocytes in the vessel lumen, consistent with a thorough washout of blood cells from the lumen. The endothelia in the microvasculature of interest were adequately stained at all levels to reveal arterioles, venules, and capillaries at different depths through the retina and choroid. We were able to trace the blood vessels through the different orders at various depths of the tissue from arteriole to venule drainage, demonstrating no breakage in the architecture of the vasculature (Figs. 2, 3, 4). The quality of the images obtained is comparable with our previously published data on fresh porcine and rat eyes.42–45 To our knowledge, this is the first report to demonstrate the intracellular cytoskeleton of the vascular endothelial cells in the intact human retinal and choroidal microvasculature.

An interesting observation was the occasional presence of capillaries crossing the fovea (Fig. 3). The presence of such capillaries in five eyes that had no history of ocular disease suggests that this may be a reasonably common occurrence. It may be that such capillaries are rarely picked up by techniques such as fluorescein angiography, trypsin digestion, or vascular casting. The presence of such capillaries in the fovea of normal eyes may require a change in the concept of a completely avascular fovea and may be relevant to many macular diseases.

The results from our choroidal perfusion are very encouraging. It is rare to be able to study the 2- and 3-D structure of the choroid and its relationship with surrounding tissues. Perfusion fixation and staining allowed us to identify the shape and intracellular structure of an individual endothelial cells as well as detailed information of cell boundary in the choriocapillaries. The 3-D relationship of the choriocapillaries, deeper choroidal vessels, Bruch’s membrane, and choroidal stroma could provide valuable information to better understand the properties of the choroid and its involvement with pathogenic diseases such as age-related macular degeneration and other retinal degenerations.

The postmortem time of these eyes ranged from 5 to more than 20 hours. However, the postmortem time appear to have no observable effect on the ability of this technique to wash out the blood, fix the tissue, or label the endothelium for microfilament and endothelium nuclei. Postmortem time may have an effect on other molecules of interest, but this is yet to be determined.

The labeling of the intact microvasculature meant that endothelium and smooth muscle cells at different levels of the vasculature can be studied in the context of the surrounding microenvironment. Intracellular structures such as endothelial F-actin and VE-cadherin and their arrangement at the arteriole, venule, and capillary levels has been clearly demonstrated (Fig. 5). Vascular smooth muscle cells have also been labeled with the phallolidin perfusion, as there is a large amount of F-actin present in these cells, enabling study of smooth muscle cell shape and arrangement at the different levels of the vascular tree.

In addition to being able to study the vasculature in a 2- and 3-D perspective, we were able to label various cell components in relation to the vasculature and study their interrelationship in a spatial context. Figure 6 showed clearly the level of cellular detail that perfusion staining and subsequent wholemount retinal immunolabeling can achieve. GFAP can be seen occurring from thick bundles running along blood vessels to very fine, seemingly singular processes meandering through the retinal tissue or fanning out from the end of the thick bundles on the vessel wall (Figs. 6B, 6D).

Immunohistolabeling of sequential thick cryosections taken at specific locations, such as the sections demonstrated in Figure 7 from the parafoveal region, will allow for multiple markers to be used to address specific questions in relation to the functional aspect of the neurovascular interaction.

Human ocular endothelium was originally considered to be an inert layer of lining cells acting as a barrier separating the nutrient-rich blood from the metabolically active neural retina and allowing exchange of nutrients and waste. Its role in normal physiological, and more recently pathologic, processes has only begun to be addressed in the past two decades. It is now known that ECs release a multitude of factors that affect surrounding cellular responses. As the ocular endothelium is the first line of contact between the systemic environment and the local neural environment, the ECs are important cells to study to gain understanding of the first changes in pathologic processes.

In addition to 2- and 3-D geographic distribution of chorioidal vasculature, more interestingly, we were able to view choriocapillaries at the cellular level. Individual ECs have been visualized in unusually large choriocapillaries (>20 μm in diameter). Information on the cell shape and intracellular cytoskeleton structure has not been reported for the human choriocapillaries. Figure 8B shows peripheral border staining with an uneven distribution and localized intracellular structures. Although the functional roles of these structural elements are unknown at this stage, they may well be shown to play a role in retinal disease.

In summary, we have demonstrated a new technique that can help us to obtain reliable data from human donor eyes. The integrated information of the spatial 2- and 3-D relationship at the cellular and subcellular level could be extremely valuable for understanding ocular physiology and disease. Although we have provided only a brief overview of the data obtained from normal eyes, the potential of such work to provide new findings related to retinal disease is evident. It may be anticipated that more detailed studies in donor eyes with a known history of retinal disease may provide valuable information regarding the pathogenesis of many retinal and choroidal diseases.

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