Microstructure and Network Organization of the Microvasculature in the Human Macula

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PURPOSE. To characterize the topography and cellular structure of the macular microvasculature using a recently developed technique of arterial cannulation, perfusion, fixation, and staining of human donor eyes.

METHODS. Sixteen human donor eyes were used. The central retinal artery was cannulated and perfused with Ringer’s, then fixative, membrane permeabilizing, and selected labeling solutions. The eyes were immersed fixed, and the retina was flattened for confocal microscopy. The macular area, including the foveola, fovea, and parafovea, was sampled. The intracellular cytoskeleton of vascular endothelial and smooth muscle cells was studied in different orders of arterioles and venules and in the capillaries. To evaluate the degree of asymmetry within vascular networks, the distribution of generation numbers and the Horton–Strahler approach to vessel naming were compared.

RESULTS. The distribution of the microvascular network in the macular region was complex but followed a general theme. The parafoveal region was supplied by dense vasculature with approximately nine closely arranged pairs of arterioles and venules. Each arteriole had abundant branches and a high degree of asymmetry (~9 generations and 3.5 orders within 1.2-mm length). Only a few arterioles (average ~2.9) supplied the terminal capillary ring. Very long spindle endothelial cells were seen in the superficial and deep capillaries. Significant heterogeneity of distribution and shape of the endothelial and smooth muscle cells was evident in different orders of the macular vasculature.

CONCLUSIONS. The authors have demonstrated for the first time the cellular structure and topographic features of the macular microvasculature in human donor eyes. (Invest Ophthalmol Vis Sci. 2010;51:6735–6743) DOI:10.1167/iovs.10-5415

The energy demand of the retina on a per gram basis has been described as higher than that of the brain. The macula is recognized as the exquisitely specialized retinal region, with high-resolution visual acuity and oxygen uptake in the macula even higher than in the remaining retina. To maintain energy-dependent processes and to clear away metabolic byproducts produced by neuronal activity, a well-regulated blood flow within the brain and retina is vital. The vascular and nervous systems communicate closely; when dysregulated, this may contribute to many important diseases. It has been demonstrated that even relatively small reductions in blood flow can have deleterious effects. The human retina is vulnerable to a wide range of retinal diseases with a vascular component and angiogenic ocular conditions, representing the leading cause of irreversible vision loss in developed countries. Such diseases include diabetic retinopathy, vascular occlusion, and age-related macular degeneration.

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 by diffusion from the retinal and choroidal vascular beds.

The macula lutea contains specialized regions, the boundaries of which are not well defined. Perhaps the clearest division of regions was developed by Hogan, who subdivided the macular region into the foveola, fovea, parafovea, and parafoveal area. The foveola corresponds approximately to the foveal avascular zone (FAZ) and includes the base of the foveal pit, where there is a peak spatial density of cone photoreceptors, for high-acuity functions. It is surrounded by terminal capillaries on the slope of the pit. In the parafoveal region, the retina has a maximum thickness because of the high densities of neural elements in all the retinal layers. Development of the retinal vasculature, particularly in the macular area, has been studied in the primates and human. Much of the literature has attempted to understand the role of retinal vasculature in macular function and diseases. However, substantial variation of topologic, morphologic, hemodynamic, and functional parameters in the retinal vascular network makes it difficult to understand and describe the properties and behavior of the vasculature, particularly in the macular area. There is still a lack of information about the structure and function of the vascular endothelium and smooth muscle cells in the macular microvasculature and no consensus on vascular network topography. Fortunately, our recently developed technique allows us to detail the spatial distribution of retinal microvasculature and its relationship to neurons and glial cells at the cellular level in human donor eyes. In the present study, we focus on the microstructure and network distribution of microvasculature in the macular area.

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 16 human eyes from 13 postmortem donors were used. All eyes were obtained from the Lions Eye Bank of Western Australia or Donate West, the West Australian agency for organ donation. Nine eyes were received after the removal of corneal buttons for transplantation. None of the donors had a known history of eye disease. Demographic data, cause of death, and postmortem time to eye perfusion for each donor are presented in Table 1. In this study we selected eyes that did not have a foveal capillary crossing the foveola to ensure that we were characterizing the more common situation in which a distinct foveal avascular zone is present.19

Preparations
Details of the method of perfusion staining of retinal microvasculature have been published previously.19 Briefly, the central retinal artery was cannulated, and residual blood was washed out with oxygenated Ringer’s solution with 1% bovine serum albumin. After the 20-minute Ringer’s wash, 4% paraformaldehyde in 0.1 M phosphate buffer was perfused for at least 1 hour for fixation. An aldehyde-based detergent, 0.1% Triton-X-100 in 0.1 M phosphate-buffered solution, was then perfused for 5 to 7 minutes to aid in the permeation of endothelial cell membranes. The detergent was washed out by a further 30 minutes of 0.1 M phosphate buffer perfusion. Then the microfilm and cell nuclei were labeled over the course of 2 hours using a mixture of phalloidin conjugated to Alexa Fluor 546 (30 U; A22283; Invitrogen, Carlsbad, CA) and cell culture reagent (bisbenzimide H 33258; 1.2 g/mL; Sigma-Aldrich, St. Louis, MO). Residual label was cleared from the vasculature by further perfusion of 0.1 M phosphate buffer, and the posterior chamber was immersion fixed in 4% paraformaldehyde overnight.

Flat Mounting of Retina
The posterior globe was dissected at the equator to allow viewing of the posterior retina. The macular region and the fovea were identified by the anatomic orientation of the globe, yellow pigmentation around the fovea, and increased choroidal pigmentation in this region. The retina was carefully dissected out without inclusion of the optic disc region. A few cuts were made to the peripheral retina to enable the retina to lie flat.

Confocal Imaging
A camera equipped with three lasers (405, 488, and 532 nm; CI; Nikon, Tokyo, Japan) was used in conjunction with a microscope (E800; Nikon). Imaging was performed with the EZ-C1 software (v.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 examined in using higher power objective lenses (×40, ×60 plan apochromatic oil lenses) for detailed imaging. Z-series were taken through a depth of 120 μm using a ×4 objective lens and up to 90 μm using a ×10 objective lens to obtain three-dimensional information on retinal microvascular architecture and to study labeled structures in the vascular endothelium and smooth muscle cells.

Topography Study
A low-magnification (×2) epifluorescent image was taken of the macular region. Then using a ×4 objective lens (Plan Apo; Carl Zeiss, Oberkochen, Germany), an optical stack was collected in an area measuring 3180 × 3180 μm square. The macular microvasculature was studied in different regions, as defined by Hogan.14 The fovea was designated to extend out to a radius of 175 μm from its center. The foveal zone extended out a further 750 μm, with the outer boundary delineated by a circle of 925 μm radius. The parafoveal area was designated to extend a further 500 μm, to lie within a circle of 1425 μm radius. Vessel orders were defined according to the convergence of smaller branches. Levels of branching were defined as generations in the vascular tree. Topologic description of trees by the Horton-Strahler and generation nomenclatures was performed in this study.20 The Horton-Strahler scheme starts at the capillary level and proceeds centripetally. The order is increased if two segments of equal order join at a bifurcation. The aim is to group vessels with similar characteristics into one order. The generation (centrifugal) scheme starts from the most central vessel considered and proceeds to the capillary level, increasing the generation by one at every branch point. The generation scheme can evaluate the degree of asymmetry within vascular networks using distributions of generation numbers in comparison with the number of different order vessels.

Study of Detailed Intracellular Microfilament Distribution
Optical sections of selected vascular segments were obtained at high magnification to study the intracellular microfilament distribution in detail. Projections of selected optical sections were made to demonstrate endothelial or smooth muscle cell microfilaments. Sketches of endothelium and smooth muscle cells were made to outline the cell shape and nucleus position.

Statistical Analysis
All statistical testing was performed using commercial software (SigmasStat, v.3.1; SPSS, Chicago, IL). For the vascular topography para-

### Table 1. Donor Eye Details and Time to Cannulation

<table>
<thead>
<tr>
<th>Donor Eye</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Eye</th>
<th>Cause of Death</th>
<th>Time to Cannulation (h)</th>
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<tr>
<td>A</td>
<td>51</td>
<td>M</td>
<td>L</td>
<td>Myocardial infarction</td>
<td>9.5</td>
</tr>
<tr>
<td>B</td>
<td>61</td>
<td>M</td>
<td>R</td>
<td>Myocardial infarction</td>
<td>10</td>
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<tr>
<td>C</td>
<td>61</td>
<td>M</td>
<td>L</td>
<td>Myocardial infarction</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>53</td>
<td>M</td>
<td>L</td>
<td>Myocardial infarction</td>
<td>19</td>
</tr>
<tr>
<td>E</td>
<td>53</td>
<td>M</td>
<td>R</td>
<td>Myocardial infarction</td>
<td>19</td>
</tr>
<tr>
<td>F</td>
<td>66</td>
<td>M</td>
<td>L</td>
<td>Cardiac?</td>
<td>14</td>
</tr>
<tr>
<td>G</td>
<td>52</td>
<td>M</td>
<td>L</td>
<td>Myocardial infarction</td>
<td>17</td>
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<tr>
<td>H</td>
<td>78</td>
<td>M</td>
<td>R</td>
<td>Sepsis</td>
<td>20</td>
</tr>
<tr>
<td>I</td>
<td>27</td>
<td>M</td>
<td>L</td>
<td>Motor vehicle accident</td>
<td>13.5</td>
</tr>
<tr>
<td>J</td>
<td>28</td>
<td>M</td>
<td>L</td>
<td>Cardiac?</td>
<td>22</td>
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<tr>
<td>K</td>
<td>43</td>
<td>M</td>
<td>R</td>
<td>Collapse, unknown cause</td>
<td>18</td>
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<tr>
<td>L</td>
<td>43</td>
<td>M</td>
<td>L</td>
<td>Collapse, unknown cause</td>
<td>18</td>
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<td>M</td>
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<td>L</td>
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<tr>
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<td>M</td>
<td>L</td>
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<td>L</td>
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<tr>
<td>P</td>
<td>53</td>
<td>M</td>
<td>L</td>
<td>Myocardial infarction(?): Motor vehicle accident</td>
<td>14</td>
</tr>
</tbody>
</table>
etters. Student’s t-testing was performed on all data that were normally distributed. The Mann-Whitney rank sum test was performed if the normality test failed for the raw data pairs. The Spearman correlation test was used to determine whether each of the measured parameters of endothelial cell and nuclei shape was correlated with vascular order in the arterial or venous network. $P < 0.05$ was taken as significant. All results are expressed as mean ± SE unless otherwise stated.

**RESULTS**

**General**

The results from this study demonstrated that when the central retinal artery was cannulated and perfused, the retinal microvasculature was well stained and the endothelial and smooth muscle cells and nuclei of the retinal vasculature were clearly labeled. They allowed us to reliably obtain detailed information of the microstructure and topography of the macular microvascularity, superimposing anatomic terminology on fundus topography.

**Topography of Macular Microvasculature**

Figure 1 shows a low-magnification projected confocal image from the macula region of the left eye of a 66-year-old donor. The retinal microvasculature had been perfusion labeled for microfilament. Optical sections were collected through 120 μm of the retina at 4-μm intervals. The area imaged was 3180 × 3180 μm square and covered the foveola, fovea, and parafovea regions, indicated by three concentric circles superimposed on the vascular network. The microvasculature network distribution appeared random; however, some common properties in topographic distribution can be found.

An arteriole was easily differentiated from a venule by its thicker wall, which takes up stronger stain with a circular pattern of smooth muscle cells. Arterioles also have slightly smaller diameters than the corresponding venules. It was also notable that the capillary free zone along the arteriole was less apparent around the macular region than in the peripheral retina, as we previously reported. There was a one-to-one relationship between a relatively large arteriole and a venule; the two were connected by a capillary plexus without the intervention of the shunt pathways present in other microcirculatory systems. We carefully traced the vascular tree in all specimens, but did not find any arteriolar-venular shunts in the macular area. No evidence was found of a capillary arcade system, as seen in peripheral retinal vessels. Neither were there connections between macular venules. Our results suggest that there is no bypass between the macular arterioles and venules or connections between pairs of arterioles or venules. Therefore, the hemodynamics of the macular vasculature appears to be mainly determined by the branching structures. In general, the arterioles were more superficial than the venules, as evidenced by the pseudocolored code properties and the overlapped relationship. Numerous pairs of arterioles and venules were found in the macular area. The average number of pairs of arterioles and venules was $8.9 ± 0.25$ ($n = 10$) arranged in a radial pattern surrounding the fovea. Only a few arterioles (on average, $2.9 ± 0.25$; $n = 10$) entered the foveal region, where they directly supplied the terminal capillary ring. The avascular region was surrounded by terminal capillaries, forming a terminal capillary ring that often had an irregular oval shape with a mean diameter of $362.3 ± 49.7 \mu m$ ($n = 7$) vertically and $410.8 ± 80.7 \mu m$ ($n = 7$) horizontally. However, there were no significant differences between the horizontal and vertical diameters ($P = 0.590$).

It was also evident that there were many bifurcations from each arteriole or venule, indicating that many generations occurred in a short segment length. Some arterioles and venules only reached the parafoveal or perifoveal region. Figure 2A shows a schematic of the nomenclature of vessel order and vessel generation. Figure 2B shows the nasal half of the macular region from the same eye shown in the area indicated by the white dashed line in figure 1. The order was increased if two segments of equal order joined at a bifurcation (i.e., where two capillaries [c] joined together to form a first-order arteriole [a-1] and two a-1 arterioles joined together to form a second-order arteriole [a-2]). However, if we use the superior arteriole from 61-year-old donor B as an example, the generation (centrifugal) scheme started from the larger retinal arteriole (Fig. 2C) and proceeded to the capillary ring, increasing the generation by one at every branch point. Five generations in the parafoveal region and eight generations in the foveal region can be counted.

Higher order arterioles give off twigs of smaller branches of capillaries and arterioles that further bifurcate to form the capillary networks before converging on the venules. The average number of vessel generations in the macular area imaged was $11.63 ± 0.40$ ($n = 32$), and there were $3.56 ± 0.14$ ($n = 29$) different orders of vessel. A significant asymmetry existed between the order and generation counts of vessels in the macular region ($P < 0.001$). The number of generations was $0.85 ± 0.04$ per 100 μm ($n = 33$) in the parafoveal region, which was significantly less than that in the foveal region ($1.24 ± 0.05$ per 100 μm; $n = 33$; $P < 0.001$). However, it was also notable that the smaller arterioles were often seen as branches in the parafoveal region.

![Figure 1](image-url)
sequent branching of the retinal arteriole into smaller arterioles (a-2 and a-1) and capillaries (c). Two capillaries (c) join together to form a first-order arteriole (a-2). The capillaries branching off the retinal arterioles are predominantly in the superficial half of the image stack (red) before connecting to the capillaries draining toward the retinal venules lying in the deeper half of the stack (green). Scale bar, 150 μm. (C) A projected image of a superior retinal arteriole traversing the parafoveal and foveal regions of the macula of a 61-year-old donor. Yellow lines: outer boundaries of the three zones as defined by Hogan.14 The retinal arteriole traversed the parafoveal region before entering the foveal region (top to the bottom) and stopped short of the foveola. Asterisk: point of branching used for counting the number of generations from this arteriole. Scale bar, 150 μm.

The capillaries that arose from the arterioles in the innermost retina often were at right angles to the vessel of origin, and the capillary meshes of the capillary plexus were relatively uniform with intercapillary anastomoses. The capillaries branching off the retinal arteriole side were predominantly in the upper half of the image stack (red pseudocolor) and then connected to the capillaries draining toward the retinal venules lying in the deeper half of the stack (green pseudocolor; Figs. 1, 2B). However, the capillaries were especially numerous in the perifoveal and parafoveal regions, diminished rapidly toward the central macular region, and were absent in the foveola. The spatial pattern and density of the macular microvascular network therefore varied greatly in different regions of the macula.

**Microstructure of Macular Microvasculature**

Microstructural heterogeneity of the macular microvasculature was studied. Figures 3, 4, and 5 show the cell shape and intracellular structure of vascular endothelium and smooth muscle cells in arterioles, capillaries, and venules, respectively. Structural information of microvessels is essential for reliable determination of the orders and generations of different components of the microvascular tree.

Figure 3 shows high-magnification confocal images of different orders of retinal arterioles (a-1 to a-4) labeled for microfilament and counterstained using Hoechst to label the nuclei. Insets highlight the peripheral border microfilament staining of individual endothelial cells of the retinal arteriole. With increasing order of retinal arteriole (i.e., upstream) the shape of the endothelium and its nuclei became more slender. There was also an increasing presence of intracellular microfilament (stress fiber) appearing as short and thick bundles that were more prominent with increasing order of arterioles and most prominent in a-4 vessels (blue arrows). Smooth muscle cells surrounding the endothelium were also present. With increasing order of the retinal arteriole, there was a thickening and widening of the microfilament bundle of the smooth muscle cells. Although only one layer of smooth muscle cells may be seen encircling the a-1 arteriole, there is a denser presence of smooth muscle cell nuclei and processes seen in the a-4 arteriole. In the larger retinal arteriole (a-4), smooth muscle cell processes may be seen overlapping other smooth muscle cell processes, suggesting more than one layer of smooth muscle cells in these larger retinal arterioles. In all orders of retinal arterioles, the smooth muscle cells had a circumferential arrangement that was perpendicular to the endothelial cell alignment.

Figures 4A and 4B are the projected images from the optical stack of the inferior area of the macula, including part of the foveola, fovea, and parafoveal region. The FAZ is uppermost. Figure 4A shows the vascular pattern and distribution of the superficial capillary network in the inner half (vitreal side) of the optical stack. The arteriole and both the side branches and the dichotomous branches came off the lateral surfaces of the arteriole and coursed horizontally in the superficial retina. Some branches then dipped into the deeper retinal tissue and anastomosed with the capillary plexus in the deeper network. The superficial capillary layer had a more three-dimensional structure in comparison with the single-layer appearance of the deep capillaries. Individual capillaries in both networks were relatively homogeneous and interconnected by anastomotic capillary bridges. There were, however, distinct differences in vascular pattern and mesh distribution between the two networks. The deep capillary network appeared to be sparser than the superficial capillary network. Examination along the z-axis of the confocal stack also highlighted the planar configuration of the deep capillary network that was similar in morphology to a two-dimensional net (Fig. 4B). Figures 4C and 4D show high-magnification confocal images of individual capillaries sampled from the superficial and deep capillary network, respectively, and insets highlighting the peripheral border staining of the endothelium. The capillary endothelium in both superficial and deep capillary networks have long peripheral border microfilament distributions running in a longitudinal.
capillary endothelial cells. In this fashion, indicating that capillaries have thin spindle-shaped endothelia, and their nuclei became more slender. There was also a thickening of the microfilament bundle of the smooth muscle cells. Although only one layer of smooth muscle cells may be seen encircling the a-1 arteriole, there is a denser presence of smooth muscle cell nuclei and processes seen in the a-3 arteriole. In the larger retinal arteriole (a-4), smooth muscle cell processes may be seen overlapping other smooth muscle cell processes, suggesting more than one layer of smooth muscle cells. In all orders of retinal arterioles, however, the smooth muscle cells had a circumferential arrangement perpendicular to the endothelial alignment. Scale bar, 50 μm.

**FIGURE 3.** Confocal images of different orders of macular arterioles labeled for microfilament. Confocal images of different orders of retinal arterioles (a-1, a-2, a-3, and a-4) were labeled for AlexaFluor546 phalloidin (red) and counterstained using Hoechst to label the nuclei (blue) and were taken with a ×60 objective lens. Insets: highlighted peripheral border microfilament staining (outlined in yellow) of individual endothelium and smooth muscle cells (red arrowheads). With the increasing order of retinal arterioles (i.e., upstream), the shape of the endothelium and their nuclei became more slender. There was also an increasing presence of intracellular microfilaments, stress fibers, appearing as short and thick bundles that were more prominent with the increasing order of the arteriole and that were most prominent in a-4 vessels. Red arrowheads: microfilament of smooth muscle cells surrounding the endothelia. With the increasing order of the retinal arteriole, there was a thickening of the microfilament bundle of the smooth muscle cells. Although only one layer of smooth muscle cells may be seen encircling the a-1 arteriole, there is a denser presence of smooth muscle cell nuclei and processes seen in the a-3 arteriole. In the larger retinal arteriole (a-4), smooth muscle cell processes may be seen overlapping other smooth muscle cell processes, suggesting more than one layer of smooth muscle cells. In all orders of retinal arterioles, however, the smooth muscle cells had a circumferential arrangement perpendicular to the endothelial alignment. Scale bar, 50 μm.

**FIGURE 4.** Confocal images of macular capillaries labeled for microfilament. (A, B) Projected images taken from the optical stack of the inferior region of the macula corresponding to the red marked region in Figure 1. The original image stack measured 62 μm in thickness and 1270 × 1270 μm in area. The superficial (A) and deeper (B) halves of the stack are shown separately. The superficial network contained arterioles (a), venules (v), and capillaries, and the deep network contained only capillaries. There was a distinctive difference in the vascular pattern, mesh density, and presence or absence of larger vessels in the two layers. The deep network had a mostly two-dimensional distribution and lower density of capillaries compared with the superficial network in which all the larger vessels were located, and the capillary distribution was more complex and had a higher density. (C, D) High-magnification confocal images of individual capillaries. Insets: peripheral border staining of the endothelium. The capillary endothelium in both the superficial (C) and the deep (D) capillaries have a long peripheral border microfilament distribution (insets, dashed yellow outline) running in a longitudinal fashion. Scale bars: 200 μm (A, B); 50 μm (C, D).

**DISCUSSION**

The number of microvessels in the human body is extremely large, including approximately 2 × 10⁹ capillaries. The microvasculature topography and complexity varies in different organs, presumably reflecting the different structural and functional features of specific tissues. The macula is a unique functional and structural region of the retina. It is specialized for diurnal high-acuity vision that requires a high density of cone photoreceptors and a large number of inner retinal cells to participate in nonconverging circuits.¹⁶ The absence of large-diameter vessels from the macula underlines its important acuity function.²³ The very center of the macula, the foveola, is presumably avascular to avoid compromising the light path to the high-density cone photoreceptor array. The present findings also indicate that specific conditions, or factors, govern vessel diameter and branching patterns in the fovea and parafovea because we observed significantly different generations of vessels in these two regions. Indeed, antiangiogenic pigment epithelium-derived factor has been shown to be highly expressed in ganglion cells at the developing fovea and most likely plays a significant role in defining the foveal vascular zone and in regulating vessel growth around the fovea.²⁴ The foveola also has a thinner inner retina, with many of the cells displaced into the foveal area. The high metabolic de-
mand of the neural retina in the macula seems likely to demand tight regulatory control to match local blood flow to tissue requirements. It is generally believed that the retina has one of the highest metabolic demands per gram of tissue in the body.\textsuperscript{4,25} We and other groups\textsuperscript{5,26,27} have demonstrated that the inner segments of the photoreceptor and the inner and outer plexiform layers have the highest metabolic demands within the retina. It is particularly interesting that cone photoreceptors have been suggested to have higher energy demands than rod photoreceptors.\textsuperscript{28,29}

It would be useful to know the detailed relationship between structural and hemodynamic heterogeneity of the microvascular networks, particularly in the macular region, in both physiological and pathologic conditions. To gain such information, there are two fundamental factors, network topology and geometric irregularities, that affect network hemodynamics. Network topology investigates how individual vessel segments are connected to one another, whereas the geometry is determined by the lengths and diameters of the vessels. Based on these two fundamental factors, it becomes feasible to interpret many complex clinical phenomena. For example, modeling of the hemodynamic characteristics of the human macula may help us estimate the hemodynamic changes in the various orders in the macular microvasculature, such as perfusion pressure, blood flow, velocity, and hematocrit. Ultimately, we hope to be able to determine the influence of vascular changes on the perfusion of the macular area. We believe that knowledge of the vascular network topography and the geometry around the FAZ is a necessary first step to understanding macular perfusion. Although extensive studies have been performed in other vascular beds, the macular vasculature seems much less uniform. As a starting point, we have focused on the distribution and branch properties that can be reliably determined from such anatomic studies.

Although considerable literature exists on this important and interesting subject, there is still no consensus about the basic relationships between the neural structure of the macula and its vascular supply.\textsuperscript{22,30} Studies in human eyes provide perhaps the most directly relevant information that is valuable for understanding retinal pathology in human retinal diseases. The purpose of the present study was to document the microstructure and network of the human macular microvasculature. Our recently developed technique allows us to study postmortem human eyes to investigate both spatial distribution and cellular structure in a great detail.\textsuperscript{59} An advantage of our preparation is that the entire retinal microvasculature can be well fixed and stained. Here we focus on the macular microvasculature, which can be studied in detail in our flat mount preparation. We believe that this report provides some of the most detailed information regarding the macular microvasculature at the topographic and intracellular level in human donor eyes. We have categorized the branching pattern of the macular microvasculature using the established centripetal Horton-Strahler approach and compared this with an analysis using the centrifugal generation scheme. This allows an assessment of the degree of branching and the asymmetry of the vascular network.\textsuperscript{20} The present study may be seen as a first step in the analysis of capillary ensembles to obtain static and planar geometric properties of the macular microvasculature.

Our results clearly demonstrated that there are abundant blood vessels supplying the macular area, with approximately nine pairs of arterioles and venules distributed in the para-

![Figures 5](image)

**TABLE 2.** EC and ECN Dimensions According to Vessel Order

<table>
<thead>
<tr>
<th>Vessel Order</th>
<th>EC Length (μm)</th>
<th>EC Width (μm)</th>
<th>Aspect Ratio of EC Length and Width</th>
<th>ECN Length (μm)</th>
<th>ECN Width (μm)</th>
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<tr>
<td>A1</td>
<td>63.6 ± 7.00 (4)</td>
<td>8.5 ± 0.80 (4)</td>
<td>7.7 ± 1.46 (4)</td>
<td>16.3 ± 1.18 (4)</td>
<td>6.4 ± 0.32 (4)</td>
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<td>A2</td>
<td>65.1 ± 4.99 (12)</td>
<td>8.8 ± 0.60 (12)</td>
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<td>14.9 ± 0.88 (12)</td>
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<td>A3</td>
<td>74.5 ± 3.68 (21)</td>
<td>9.4 ± 0.50 (21)</td>
<td>8.3 ± 0.61 (21)</td>
<td>15.2 ± 0.46 (20)</td>
<td>6.4 ± 0.32 (20)</td>
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<td>A4</td>
<td>96.6 ± 2.53 (14)</td>
<td>8.6 ± 0.46 (14)</td>
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<td>V1</td>
<td>62.5 ± 8.16 (6)</td>
<td>8.8 ± 0.44 (6)</td>
<td>7.1 ± 0.84 (6)</td>
<td>14.1 ± 1.32 (6)</td>
<td>7.1 ± 0.84 (6)</td>
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<tr>
<td>V2</td>
<td>64.6 ± 6.66 (7)</td>
<td>10.8 ± 1.01 (7)</td>
<td>6.2 ± 0.72 (7)</td>
<td>16.3 ± 1.42 (7)</td>
<td>8.5 ± 0.68 (7)</td>
</tr>
<tr>
<td>V3</td>
<td>74.5 ± 4.03 (26)</td>
<td>15.4 ± 0.66 (26)</td>
<td>4.9 ± 0.24 (26)</td>
<td>14.8 ± 0.54 (26)</td>
<td>9.2 ± 0.39 (26)</td>
</tr>
<tr>
<td>V4</td>
<td>72.1 ± 3.67 (24)</td>
<td>21.6 ± 1.11 (24)</td>
<td>3.5 ± 0.22 (24)</td>
<td>14.4 ± 0.56 (24)</td>
<td>12.5 ± 0.55 (24)</td>
</tr>
</tbody>
</table>

Values are mean ± standard error (sample number). EC, endothelium; ECN, endothelium nuclei.
vacular regions. Rapid branching results in a relatively short distance (few hundred microns) of capillary between the arteriolar and venule. We have found that the arterioles in the macular area have a high number of generations (~10) within a very short length (~1.2 mm) but relatively few orders (3–4) when compared with other vascular beds.\(^3\)\(^9\) There were typically only two to four pairs of arterioles and venules found in the foveola region supporting the terminal capillary ring. The venular and arterial portions of the macular vasculature were closely associated. All these features create a high density of capillaries in the parafoveal region and a lower density of capillaries in the fovea and foveal avascular zone. The capillary network appears to be dense and three dimensional, particularly in the parafoveal area.

The central role of the cardiovascular system is to maintain an adequate capillary flow.\(^2\)\(^0\) Perfusion and metabolic activity are heterogeneous and spatially correlated. It is difficult to examine the effects on flow distribution and oxygen level in terminal vascular beds of inherent irregularity in structure, hemodynamic properties, and local variations in oxygen demand.\(^3\)\(^2\) Mean macular capillary flow velocities have been found to range from 1.37 to 3.3 mm/s.\(^5\)\(^2\)\(^3\)\(^4\)\(^7\)\(^8\) Apparently, macular capillary flow velocity is comparable to that in the brain, which has been reported to range between 0.3 and 3.2 mm/s,\(^5\)\(^5\) and higher than that in the mesentery, with a mean velocity of 0.8 mm/s.\(^2\)\(^0\) Based on these flow velocity values and short lengths of capillaries in the macula, blood transit time in an individual capillary would be well under 1 second.

To our knowledge, the intracellular cytoskeleton of human macular microvasculature has not been reported before. Knowledge of the cellular structure of the macular vasculature helps to reliably determine the topologic features by confirmation of the vessel orders. It is also valuable for understanding macular physiology and pathology.

Endothelial cells are subjected to an extremely dynamic range of mechanical stresses, including fluid shear, hydrostatic pressure, and cyclical stretch. Endothelial cells are sensitive to such stress and modify their cytoskeletal composition and architecture according to the different magnitude and type of stress experienced. The effect of shear stress on endothelial cell morphology and function has been extensively studied in cultured cells under different shear stress conditions and in vivo at different regions where local blood flow patterns are predictable.\(^3\)\(^6\)\(^–\)\(^8\)\(^9\)\(^0\)\(^1\)\(^0\)\(^1\)\(^0\) Those results indicate that under high fluid shear stress, endothelial cells tend to elongate and orient themselves along the flow direction. In addition, stress fibers of actin filaments become more prominent and align along the cell long axis. Endothelial cells undergo profound morphologic changes in response to alterations in shear stresses that are imposed on them by blood flow, and these responses have important implications for physiologic and pathophysiologic function of blood vessels.

Our results suggest that there is reasonably high blood velocity and shear stress in the A-\(\lambda\) vessels, evidenced by the long spindle shape of the endothelial cells and the presence of intracellular stress fibers in the endothelium as previously reported in larger retinal arterioles.\(^3\)\(^9\)\(^–\)\(^4\)\(^2\)\(^2\) Our results indicate there are some special features in the macular hemodynamics. Our growing knowledge of the roles and regulation of cytoskeletal components in endothelium and further data from macular hemodynamics may provide us with an improved understanding of endothelial function in both health and disease.

Like most terminal vascular beds, macular vasculature is expected to have a high degree of structural heterogeneity and is typically asymmetric and irregular. The topological structure of the networks was analyzed using the Horton-Strahler technique and a generation scheme. The Horton-Strahler nomenclature provides only a rather coarse classification, and the topological structures resulting from this approach are therefore likely to be approximate but to contain some branch properties. Generation numbers were assigned to the vessel segments on the basis of the number of bifurcations, reflecting the topological structure of the network more accurately than order numbers. Most macular arterioles were found to have dichotomous branching structures that could be symmetric or asymmetric. The present study sought to obtain information of both order number and generation number for the macular vasculature.

The human donor eyes selected for this study were obtained from donors with no previous history of ocular disease. The data obtained, therefore, are presumably representative of the normal human eye. The retina lends itself well to a study of vascular patterns. Perhaps no other tissue in the body can be removed with such a minimum of dissection, providing a great opportunity for us to study macular microvasculature in intact macular tissue. The endothelial cells in the microvasculature of interest were adequately stained at all levels to reveal arterioles, venules, and capillaries at different depths through the macula. We were able to trace the blood vessels through the different orders at various depths of the tissue from arteriole to venule. The quality of the images obtained is comparable with our previously published data using fresh porcine and rat eyes.\(^5\)\(^9\)\(^–\)\(^4\)\(^2\)\(^2\)\(^2\)\(^2\) 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 structure such as endothelial f-actin and their arrangement at arteriole, venule, and capillary levels has been clearly demonstrated (Figs. 3–5). There are several limitations in the measuring of the shape of the endothelial cells, particularly in smaller vessels. Identification of the endothelial cell border is dependent on peripheral border staining, which does not stain as strongly as do other membrane stains. The endothelium is slender throughout the microvasculature and tends to curl around the vascular lumen. Because the measurements were done on a two-dimensional projection of optical sections, it was often difficult to find endothelium that has its whole cell border within the plane of the image to allow accurate measurements.

Vascular smooth muscle cells have also been labeled with phalloidin perfusion because there is a large amount of f-actin present in these cells, enabling the study of smooth muscle cell shape and arrangement at the different levels of the vascular tree. In addition to the roles of endothelial cytoskeleton we reported previously,\(^5\)\(^9\)\(^–\)\(^4\)\(^2\)\(^2\)\(^2\)\(^2\) there are a number of interesting features in the macular vasculature. First, a long spindle shape of the endothelial cells has been found in the both superficial and deep capillaries in the foveal and parafoveal regions. This may be related with their high exchange capability. Second, we were able to precisely define the relationship between the terminal arterioles, venules, and capillaries based on shape, size, distribution, and structure of the endothelium and smooth muscle cells. Interestingly, the terminal arterioles are always very close to each other and also close to the venules, thus minimizing the length of the capillaries. Perhaps this vascular pattern is to provide sufficient capillary perfusion and high regulatory capability to adapt to functional demands of the high density and functional requirements of the macular neurons.

Our results demonstrated a high degree of structural heterogeneity and typically asymmetric and irregular distribution in the macular vasculature. Significant structural heterogeneity is inevitable, particularly in the macular region, because network structures must continually adapt to growth and changing functional demands. Local addition or
removal of individual segments would result in a loss of symmetry. In addition to the topological data of this study, future work is required to better understand the geometric properties of the macular microvasculature, such as lengths and diameters of the segments as well as capillary density. It is predictable that the relationship between structural (topological and geometric) and hemodynamic heterogeneity in the microvascular network of the macula would be more complicated than other tissues and would have different properties in specific regions such as the fovea and the parafovea. Topological heterogeneity (varying numbers of vessel segments) indicates different pathways and variations in pathway flow resistance. Geometric heterogeneity (variation of segment length and diameter) results in differences in flow resistance between alternative pathways. Future work will attempt to quantify the contributions of these heterogeneities to hemodynamic properties of microvascular networks and perform corresponding simulations. Our overall goal is to understand and predict the relationship between microvascular structure and tissue function in the normal macula and various forms of macular disease.

In summary, we have documented some topographical and cellular structure properties of the human macular microvasculature that could be extremely valuable for understanding macular physiology and pathology. It has been increasingly recognized that both blood vessels and nerves are vital channels to and from tissues and have much more in common than was originally anticipated. From a developmental viewpoint, they use similar signals and principles to differentiate, grow and to navigate toward their targets. They also contribute to physiological regulation and induce pathologic insults when dysregulated. It is understandable that a delicate balance between special macular microvasculature and high-density neurons requires comprehensive mechanisms to maintain healthy macular function.

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References


