Three-Dimensional Microscopy Demonstrates Series and Parallel Organization of Human Peripapillary Capillary Plexuses

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Oxygen consumption within the retina, per unit weight of tissue, is significantly greater than most other organs in the human body.1 The retinal circulation plays a major role in oxygen delivery and is responsible for sustaining the homeostatic mechanisms that underlie visual function.2 Unlike other organs, the angioarchitectural characteristics of the retinal circulation are unique, being confined by the optical properties of the eye and the heterogeneous profile of energy consumption within the layered retina.3 From a biophysical standpoint, the concentration of vascular structures within the retina can be increased by only a finite amount before the optical pathway between the light source and photoreceptors is disturbed. Similarly, a reduction in vascular density may serve to provide a clearer image of the visual field to photoreceptors but elevates the risk of retinal ischemia during states of increased energy consumption. Understanding the organization and topologic properties of the retinal circulation is, therefore, of great importance for defining the vasculogenic mechanisms that support retinal physiology as well as understanding the selective vulnerability of neuron-glial populations to retinal vascular diseases.

Previous reports have shown that the retinal circulation is ramified and spatially specialized.4–7 Histologic data from humans4,5,8,9 and other mammalian species6,7 have demonstrated great variations in the density and laminar organization of the superficial and deep retinal capillary networks. To date, two-dimensional topologic information combined with established geomorphologic classifications, such as the Horton-Strahler nomenclature and fractal analysis, have been the basis for conductance, pressure, and flow computations within retinal vessels.10 However, there is increasing evidence to suggest that reliable interpolation of the hemodynamic properties of a vascular bed requires three-dimensional geometric information rather than considering a vascular network as a two-dimensional construct.11,12 Such approaches to study the brain have provided invaluable insights into the sites of greatest hemodynamic resistance and pressure change in the cortical circulation.13 To our knowledge, similar detailed

Keywords: retina, capillary, microcirculation, optic disk, vascular disease
investigations of the human retina are lacking but are required to facilitate future important studies on multiscale modeling of blood flow in the retinal circulation. Such angioarchitectural mapping studies are also expected to facilitate detailed investigations and insights into pathogenic mechanisms that underlie important vascular diseases, such as macula telangiectasia type 2, retinal vein occlusion, and paracentral acute middle maculopathy.

This report is a detailed characterization of the normal human retinal circulation by using two-dimensional and three-dimensional histologic imaging techniques. We focus on the peripapillary retina and define the morphologic and quantitative properties of the capillary plexuses within this region. The organization of conduits that interconnect capillary beds as well as the morphologic patterns of vascular structures that connect the capillary system to precapillary arterioles and postcapillary venules are documented. This study provides new information about retinal vascular physiology and may provide an anatomic basis for understanding the variable natural course of retinal vascular diseases. The data contained in this report also serve as an important histologic correlate for interpreting in vivo imaging techniques, such as optical coherence tomographic angiography, that permit two-dimensional and three-dimensional visualization of the retinal circulation.

**Materials and Methods**

The 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 Tissue Preparation**

Human donor eyes used in this report were obtained from DonateLife WA, the organ and tissue retrieval authority in Western Australia, Australia. Five eyes from four normal Caucasian female human donors were used (age range, 20–74 years old). Donor eyes used for this research had no documented history of eye disease. The demographic details and medical comorbidities of donor eyes are presented in Table 1.

<table>
<thead>
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<th>Donor</th>
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<th>Cause of Death</th>
<th>Sex</th>
<th>Age, y</th>
<th>Time, h</th>
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<tbody>
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<td>R &amp; L</td>
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<td>F</td>
<td>75</td>
<td>4</td>
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<tr>
<td>2</td>
<td>L</td>
<td>Sepsis</td>
<td>F</td>
<td>74</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>L</td>
<td>Leukemia</td>
<td>F</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>Cancer</td>
<td>F</td>
<td>67</td>
<td>9</td>
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</table>

L, left; R, right; F, female.

<table>
<thead>
<tr>
<th>Demographic and Clinical Details of Human Donor Eyes</th>
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</table>

Images of the optic disk and peripapillary regions were captured using a confocal scanning laser microscope (Nikon Eclipse 90i; Nikon Corporation, Tokyo, Japan, or Nikon Eclipse E800; Nikon Corporation). The superior, temporal, inferior, and nasal quadrants were imaged using a Nikon 10× NA 0.45 dry objective lens that had a field of view of 1.27 mm × 1.27 mm (Fig. 1). Additional imaging was acquired using a Nikon 20× NA 0.6 objective lens and Nikon 40× NA 1.0 oil objective lens. Using a motorized stage, a series of z-stacks were captured for each specimen beginning from the vitreal surface, at the level of the inner limiting membrane, to the outer nuclear layer. Each z-stack consisted of a depth of optical sections, 1 μm apart, along the z-axis. Sections of the eyes labeled with lectin FITC/phalloidin TRITC were visualized via 488-nm/561-nm argon laser excitation with emissions detected through 515-nm/605-nm band pass filters, respectively. Simultaneous scanning was done in each donor eye to visualize the nuclei by using 488-nm argon laser excitation with emissions detected through a 450-nm band pass filter.

Areas where we chose to study capillary branching patterns were imaged with the Nikon 40× NA 1.0 oil objective lens. In these regions, tissue extending from the inner limiting membrane to the outer nuclear layer was imaged at 1-μm step sizes along the z-axis.

**Confocal Scanning Laser Microscopy**

**Image Analysis**

Confocal image files were processed with Imaris (Bitplane, Zurich, Switzerland) and/or ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA). Two-dimensional and three-dimensional images of each confocal stack were generated. The brightness and contrast were adjusted using the “contrast change-linear stretch” function on Imaris (Bitplane) to ensure that the margins of vessels could be visualized clearly. Arterioles, capillaries, and venules in the peripapillary regions were characterized in confocal microscope images by using the morphologic definitions provided in previous reports. Retinal arterioles and venules were distinguished by their dichotomous branching patterns and the presence of a vascular-free zone that was more prominent adjacent to...
arterioles than venules. Capillaries were distinguished by their loop patterns, multilayered organization, tortuous trajectory, and absence of an immediate vascular-free zone.

**Two-Dimensional Image Analysis.** Confocal volumes acquired using the 10× lens were used for this part of the analysis. The peripapillary circulation was stratified into different capillary plexuses based on vascular morphology and their location relative to the nuclear layers of the retina (Figs. 1, 2). Two-dimensional images (n = 80), generated by projecting all confocal slices that composed a capillary plexus, were used to attain the below measurements by using our previously published criteria:4,5,19

1. **Capillary diameter:** Defined as the perpendicular distance across the maximum chord axis of each vessel. Each capillary plexus image was partitioned into nine equal regions, and measurements were obtained from each region to ensure representative sampling. Five capillaries were measured in each region, resulting in
45 measurements per image, and the average of these 45 measurements was used in the analysis.

2. Capillary density: This parameter was reflected by measurements including intercapillary distance. A 3 x 3 grid consisting of two equally spaced horizontal and two equally spaced vertical perpendicular line segments measuring 0.5 mm each were superimposed over images. The manual counts of capillary intersections across the four-line segments were recorded. For each two-dimensional image, the intercapillary distance was derived by dividing the total number of capillary intersections across the four-line segments by the total length covered by the four-line segments (2 mm).

3. Capillary segment length: Defined as the distance from one bifurcation point of a capillary to another bifurcation point. The lengths of five complete capillary segments from each of the 80 two-dimensional images (20 images from each plexus) acquired with the 10X lens were measured.

Three-Dimensional Image Analysis. Three-dimensional images (n = 78), constructed using confocal volumes acquired with the Nikon 40X NA 1.0 oil objective lens (field of view of 318.5 μm x 318.5 μm), were used to evaluate vascular branching patterns and connections between capillary beds, precapillary arterioles, and postcapillary venules. By using the Imaris software (Bitplane), confocal volumes were rotated in the x-, y-, and z-axes to precisely delineate the topologic characteristics of capillary beds and their connections to each other as well as feeding arterioles and draining venules. Images depicting two or three different angles of rotation of the confocal volume were used to illustrate branching patterns. The length and diameter of interconnecting vessels between the (1) superficial capillary plexus (SCP) and intermediate capillary plexus (ICP) and (2) ICP and deep capillary plexus (DCP) visible in the three-dimensional images (n = 78) acquired using the 40X lens was measured using Imaris software (Bitplane). Two vessels connecting the SCP and ICP (n = 156) and two vessels connecting ICP and DCP (n = 156) were identified from each of the 78 samples for measurement. All images in this manuscript were prepared using Adobe Photoshop CS5 (version 12.1; Adobe Systems, Inc., San Jose, CA, USA) and Adobe Illustrator CS5 (version 15.1.0; Adobe Systems, Inc.).

Statistical Analysis
Data was analyzed using SigmaPlot (version 12.0; SPSS, Chicago, IL, USA). Three-way ANOVA was used to compare measurements between capillary plexuses and the four quadrants surrounding the optic disk. A P value less than or equal to 0.050 was considered significant. Results are expressed as mean ± standard error.

RESULTS
Morphologic Characteristics of the Peripapillary Capillary Plexuses
In all four quadrants that were studied, the peripapillary microvasculature was seen to be composed of four capillary plexuses as follows (Figs. 1, 2):

1. Radial peripapillary capillary plexus (RPC; Fig. 2B): This plexus was colocalized to the nerve fiber layer (NFL; Fig. 1B) and characterized by straight, long capillary segments that were predominantly oriented parallel to each other. Long capillary segments were interconnected by diagonal and orthogonally oriented shorter segments. Radial peripapillary capillaries commonly formed hairpin turns along the x-y plane. Some hairpin turns formed closed loops, whereas the majority formed open loops (Fig. 3). Radial peripapillary capillaries followed either a “Y” or “H” branching pattern (Fig. 3). Out of 106 branch points that were studied, 42% were found to have the “H” configuration and 58% were found to have the “Y” configuration.

2. Superficial capillary plexus (Fig. 2C): The SCP was colocalized to the level of the retinal ganglion cells (Fig. 1B) and characterized by straight, long capillary segments that were predominantly oriented parallel to each other. Long capillary segments were interconnected by diagonal and orthogonally oriented shorter segments. Radial peripapillary capillaries commonly formed hairpin turns along the x-y plane. Some hairpin turns formed closed loops, whereas the majority formed open loops (Fig. 3). Radial peripapillary capillaries followed either a “Y” or “H” branching pattern (Fig. 3). Out of 106 branch points that were studied, 42% were found to have the “H” configuration and 58% were found to have the “Y” configuration.
were frequently more superficial in position than the retinal veins. Capillaries composing the SCP were derived directly from precapillary arterioles and drained directly into postcapillary venules.

3. Intermediate capillary plexus (Fig. 2D): This plexus was characterized by a three-dimensional meshwork of vessels that was more dense and tortuous than the other capillary plexuses. It was localized to the superficial part of the inner nuclear layer (INL) and the deep part of the inner plexiform layer (Fig. 1B). The ICP was composed of vertical and oblique capillary segments that adjoined vessels in the SCP above and the deep capillary layer below. Segments of retinal arterioles and venules were frequently seen at the level of the ICP, but we did not identify any direct communications between these arterioles/venules and the capillaries of the ICP.

4. Deep capillary plexus (Fig. 2E): The DCP was localized to the boundary of the INL and outer plexiform layer (OPL; Fig. 1B). The DCP was largely flat and planar in configuration and characterized by numerous closed vascular loops. Segments of retinal arterioles and venules were not seen at the level of the DCP.

Quantitative Properties of the Peripapillary Capillary Plexuses

The mean capillary diameter and intercapillary distance measurement of each plexus are provided in Table 2. The mean capillary diameter for all plexuses was 8.89 ± 0.14 μm (n = 80), and the mean intercapillary distance was 50.25 ± 1.52 μm (n = 80). There was no difference in mean capillary diameter or intercapillary distance between the nasal, temporal, inferior, and superior quadrants of the optic nerve head for all four plexuses (all P > 0.050).

Comparisons of intercapillary distance between the different plexuses are shown in Figure 4A. There was a significant difference in the intercapillary distance between the different plexuses (P = 0.011). The mean intercapillary distance in the RPC was significantly lower than the ICP and DCP (both P < 0.008). The mean intercapillary distance in the SCP was also significantly lower than the ICP and DCP (both P < 0.010). The mean intercapillary distance in the DCP was significantly lower than the ICP (P = 0.012).

Comparisons of capillary diameter between the different plexuses are shown in Figure 4B. There was a significant difference in capillary diameter between the different plexuses (P = 0.001). The mean capillary diameter in the RPC was significantly greater than the ICP and DCP (both P < 0.001). The mean capillary diameter in the SCP was also greater than the ICP and DCP (both P < 0.002). There was no difference in mean capillary diameter between the RPC and SCP (P = 0.969) nor the ICP and DCP (P = 0.756).

The mean capillary segment length for all plexuses was 313.5 ± 6.4 μm (n = 400). The mean capillary segment lengths for individual plexuses are shown in Table 3 (n = 400).

### Table 2. Intercapillary Distance and Capillary Diameter of the Four Peripapillary Capillary Plexuses in Normal Eyes

<table>
<thead>
<tr>
<th>Peripapillary Capillary Plexus Type</th>
<th>Intercapillary Distance, Mean ± SE, μm</th>
<th>Capillary Diameter, Mean ± SE, μm</th>
</tr>
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<tr>
<td>All</td>
<td>50.25 ± 1.52</td>
<td>8.89 ± 0.14</td>
</tr>
<tr>
<td>Radial peripapillary capillary plexus</td>
<td>47 ± 1.52</td>
<td>9.8 ± 0.18</td>
</tr>
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<td>Superficial capillary plexus</td>
<td>47.5 ± 1.60</td>
<td>9.65 ± 0.11</td>
</tr>
<tr>
<td>Intermediate capillary plexus</td>
<td>54 ± 1.38</td>
<td>8.02 ± 0.12</td>
</tr>
<tr>
<td>Deep capillary plexus</td>
<td>52.5 ± 1.40</td>
<td>8.12 ± 0.19</td>
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### Table 3. Capillary Segment Length in Each of the Four Peripapillary Capillary Plexuses in Normal Eyes

<table>
<thead>
<tr>
<th>Peripapillary Capillary Plexus Type</th>
<th>n</th>
<th>Mean ± SE, μm</th>
</tr>
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<tbody>
<tr>
<td>All</td>
<td>400</td>
<td>313.5 ± 6.40</td>
</tr>
<tr>
<td>Radial peripapillary capillary plexus</td>
<td>100</td>
<td>335 ± 8.24</td>
</tr>
<tr>
<td>Superficial capillary plexus</td>
<td>100</td>
<td>319 ± 5.48</td>
</tr>
<tr>
<td>Intermediate capillary plexus</td>
<td>100</td>
<td>280 ± 3.89</td>
</tr>
<tr>
<td>Deep capillary plexus</td>
<td>100</td>
<td>320 ± 8.01</td>
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Table 4. Length and Diameter of Interconnecting Vessels Between SCP and ICP and ICP and DCP

<table>
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<tr>
<th>Measurement</th>
<th>SCP and ICP, Mean ± SE, μm</th>
<th>ICP and DCP, Mean ± SE, μm</th>
<th>P Value</th>
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<td>Length</td>
<td>37.78 ± 0.12</td>
<td>32.81 ± 0.12</td>
<td>&lt;0.001</td>
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<tr>
<td>Diameter</td>
<td>9.49 ± 0.17</td>
<td>9.85 ± 0.11</td>
<td>0.34</td>
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</tbody>
</table>

* n = 156.

Significant differences were found between the RPC and the other three plexuses (SCP, ICP, and DCP), as well as the ICP and the other three plexuses (RPC, SCP, and DCP), (P < 0.001).

Three-Dimensional Analysis of Interconnecting Vessels Between Plexuses

The mean lengths and diameters of interconnecting vessels between the SCP and ICP (n = 156) as well as the ICP and DCP (n = 156) are shown in Table 4. There was a significant difference in lengths but not in diameters of interconnecting vessels between the SCP and ICP versus the ICP and DCP (P < 0.001).

Three-Dimensional Analysis of Capillary Inflow Pathways

In the four quadrants of all eyes, precapillary arterioles were always seen to be connected to capillaries composing the SCP. We did not find any examples where precapillary arterioles connected directly to capillaries composing the RPC, ICP, or DCP. A total of 35 branch points in the SCP were randomly chosen to define the various patterns of vascular connections between the SCP and surrounding capillary beds. In order to ensure that the arterial side of the capillary circulation was evaluated, we examined the first branch point of the SCP. The pathway and trajectory of capillary segments were precisely determined by rotating confocal volumes in the x-, y-, and z-axes. We found it difficult to isolate the trajectory of individual capillary segments when two-dimensional image evaluation strategies (such as projection of the confocal stack to a single image or scrolling through the confocal volume and examining confocal slices individually) were solely applied. Such strategies made it difficult to accurately separate the trajectory of individual capillary segments from overlying or underlying vascular structures.

The frequencies of the different branching patterns of the SCP have been schematically illustrated in Figure 5, with representative images of individual branching patterns shown in Figures 6 through 9. A total of four different branching patterns were identified and are as follows:

- Pattern 1: This was the most common pattern (62.8%, n = 35) and was characterized by bifurcation of the capillary segment such that one branch connected with the RPC and the second branch remained in the plane of the SCP (Fig. 6).
- Pattern 2: Seen in 22.8% (n = 35) of cases and characterized by bifurcation of the capillary segment such that one branch connected with either the ICP or DCP while the second branch remained in the plane of the SCP (Fig. 7).
- Pattern 3: Characterized by bifurcation of the capillary segment such that one branch connected with the RPC and the second branch connected with either the ICP or DCP. This was seen in 8.6% (n = 35) of cases (Fig. 8).
- Pattern 4: The least common pattern (5.8%, n = 35) and characterized by trifurcation of the capillary segment to send a branch to the RPC, a second branch that connected with either the ICP or DCP, and a third branch that remained in the plane of the SCP (Fig. 9).

Connecting vessels between the SCP and surrounding capillary plexuses were typically oblique and tortuous in their trajectory, with only a few short, vertical intercapillary connecting segments seen. Radial peripapillary capillaries (Fig. 10) and capillaries of the deeper plexuses (Fig. 11) were noted to receive more than one connecting branch from the SCP. Direct connections between the RPC and capillaries of the ICP and DCP were not seen. A total of 40% of capillaries in the ICP were seen to have a connecting branch to vessels of the DCP.

Three-Dimensional Analysis of Capillary Outflow Pathways

In all eyes, postcapillary venules were always seen to be connected to the SCP. We did not find any evidence where the postcapillary venules were directly connected to the RPC, ICP, or DCP. A total of 43 branch points in the terminal portion of the SCP were studied. In order to ensure that the venous side of the capillary circulation was studied, we examined the final branch point within the SCP prior to connecting to the draining venule.

The frequencies of the different outflow patterns of capillaries in the SCP have been schematically illustrated in Figure 5, with representative images of individual branching patterns shown in Figures 12 through 15. A total of different four branching patterns were identified and are as follows:
Pattern 1: Seen in 41.9% \((n = 43)\) of cases and characterized by a capillary of the RPC and a capillary in the same plane of the SCP connecting with a single capillary segment of the SCP (Fig. 12).

Pattern 2: Characterized by a capillary of the ICP or DCP and a capillary in the same plane of the SCP connecting with a single capillary segment of the SCP (Fig. 13). This was seen in 37.2% \((n = 43)\) of cases.

Pattern 3: Characterized by a branch of the RPC and a branch of the ICP or DCP connecting with a single capillary segment of the SCP (Fig. 14). This was seen in 11.5% \((n = 43)\) of cases.

Pattern 4: The least common pattern \((9.4\%, n = 43)\) and characterized by a branch of the RPC, a second branch that connected with either the ICP or DCP, and a third branch that remained in the plane of the SCP connecting with a single capillary segment of the SCP (Fig. 15).

Radial peripapillary capillaries (Fig. 10) and capillaries of the deeper plexuses (Fig. 11) were noted to have numerous outflow connector channels with the SCP. Direct outflow connections between the RPC and capillaries of the ICP and DCP were not seen.

**DISCUSSION**

This study provides a detailed account of the two- and threedimensional angioarchitectural characteristics of the human peripapillary microvasculature. The different vascular configurations that facilitate communication between capillary beds as well as predominant inflow and outflow vascular channels within the retina have been defined and demonstrated in order to better understand the vasculogenic mechanisms that govern retinal health and disease. The peripapillary circulation was chosen as the region of interest as it has been less well studied than other retinal eccentricities and is a critical site in the...
We show that the human peripapillary microvasculature is composed of four capillary plexuses that are spatially localized to the following retinal layers: (1) NFL, (2) retinal ganglion cell layer (GCL), (3) inner border of the INL and outer border of the inner plexiform layer, and (4) outer border of the INL and inner border of the OPL. Such an organization is similar to the peripapillary microvasculature of the pig eye, as previously described by Rootman. Consistent with previous investigations, this study provides evidence of morphologic specialization of retinal capillary plexuses that, like the brain, may be related to the unique metabolic demands of the cellular environment they traverse. In the gray matter of the brain, there is a significant relationship between capillary density and rates of energy consumption; such a relationship may also occur in the retina where the profile of oxygen consumption is known to be heterogeneous.

A number of studies have examined the relationships between structural and hemodynamic heterogeneity in the brain, eye, skeletal muscle, and gastrointestinal tract; however, they predominantly considered the vascular bed as a two-dimensional construct. Fraser et al. modeled oxygen transport in discrete microvascular volumes by using three-dimensional reconstructed capillary networks and compared these results to equivalent parallel capillary arrays. They showed that the profile of oxygen delivery ascertained by the two models are not equivalent and by doing so, exemplified the importance of considering the three-dimensional characteristics of a vascular system in microcirculatory studies. Similarly, Gould et al. used vectorized data of multiscale cortical vascular morphology to investigate sites of greatest...
hemodynamic resistance in the cortical circulation. Using simulations of blood flow, hematocrit, and oxygen tension, they showed that marked hemodynamic variations in randomly organized capillary beds are responsible for uniform cortical tissue perfusion and oxygenation. They also concluded that measurements in the three-dimensional scale are required for meaningful characterization of microcirculatory flow patterns in the brain. A major strength of the present report is that it uses three-dimensional image analysis and volume rendering to define the topologic characteristics of the retinal circulation. Doing so has allowed us to expand our understanding of retinal vascular morphology.

The variations in microcirculatory morphology between retinal layers may reflect the need for regional specializations of the neurovascular unit within the retina. The retinal neurovascular unit is composed of vasculature (namely the capillary endothelia), neurons, glial cells, pericytes, and smooth muscle cells. Each component of the neurovascular unit is intrinsically linked to the other and collectively serves to fine-tune the supply of nutrients and substrates as well as coordinate the removal of wastes and by-products. The overriding purpose of the neurovascular unit is to optimize regional metabolic efficiency (i.e., to increase blood flow to sites of increased cellular activity and to preserve flow at sites of low activity). A key role of the neurovascular unit is to regulate oxygen delivery to retinal cells. In this study, we found that capillary diameter was significantly lower in the intermediate and deep plexuses compared with other networks. A reduction in capillary diameter increases the surface area to blood volume ratio, resulting in a greater exchange of oxygen and other metabolites for a given volume of blood. This implies that the ICP and DCP are sites where red blood
cell velocity is reduced in order to facilitate greater oxygen extraction from surrounding cells. This may be one example where regional specialization of the neurovascular unit serves to increase the efficiency of oxygen extraction. Additionally, intercapillary distance has an important relationship to oxygen diffusion properties, and it has been reported that a reduction in intercapillary distance results in decreased oxygen diffusion times, leading to increased energy production.26 Intercapillary distance was lowest in the RPC network, suggesting that oxygen diffusion may be an important mechanism by which neuronal function is supported in the NFL, a regional specialization that may be required to support the large nonmyelinated axons of the ganglion cells, which have very high metabolic demands required for ion pumping, similar to the gray matter in the brain.23

In addition to optimizing oxygen delivery, the neurovascular unit is expected to serve a critical role in regulating other physiologic properties of the retina, such as fluid balance within the extracellular space. In a recent publication, Spaide27 applied Poiseuille’s law of flow to the geometric properties of the retinal circulation to propose that net fluid pressure in the deep plexus of the retina is lower than the superficial plexus. He also discussed the concept of convection flow through the interstitium of the retina, from the superficial plexus to the deep plexus, due to the differences in osmotic and hydrostatic forces acting across the capillary walls of these two circulations and postulated that Müller cells were key players in regulating hydration within the retinal interstitium, similar to the role played by astrocytes in controlling bulk flow within the brain parenchyma.27 In this study we show that the ICP has

Figure 9. Type 4 branching pattern of the SCP. This was characterized by trifurcation of the superficial capillary segment such that one branch connected to the RPC, a second branch connected with either the ICP or DCP and a third branch that remained in the plane of the SCP. Three-dimensional constructions of a confocal volume, presented at three different angles (A-C), highlight the branching pattern. The branch point of interest is denoted by the inset on panel A. Trajectories of relevant capillary segments are highlighted by false-colored overlays. A schematic illustration of this branching pattern is provided in the right panel. Precapillary arterioles are marked on each image are displayed as a single image is shown in panel A.
a highly three-dimensional organization composed of vertical
and oblique looping capillary segments and speculate that this
circulation serves an important role in regulating fluid balance
within the retinal interstitium separating the superficial and
depth plexus. We propose that one function of the ICP in the
retina may be analogous to the role served by the vasa recta
capillary system of the kidney in maintaining the medullary
interstitial gradient. Experimental studies have shown that
the capillaries of the vasa recta are highly permeable to solute
and water. This circulation, therefore, not only provides
nutrients and oxygen to the medullary portion of the nephron
but also plays an important role in controlling the osmolality
and ionic composition of the medullary nephron segments.

The vasa recta reduces the amount of solute washout from the
medullary interstitium by acting as a countercurrent exchang-
er. In the descending limb, blood in the vasa recta becomes
increasingly hyperosmolar due to the outward diffusion of
water and the inward diffusion of solute. The converse occurs
in the ascending limb. It is known that regulating blood flow in
the vasa recta is an important mechanism by which the kidney
controls the medullary interstitial gradient and, by extension,
the urine concentration. Pericyte-mediated vasoconstriction
is an important mechanism by which blood flow is regulated in
vasa recta capillaries. As the ICP is located within the stratum

**FIGURE 10.** Inflow and outflow connections between the radial peripapillary capillaries and the SCP occurs at multiple branch points. Three-
dimensional constructions of a confocal volume, presented at two different angles, illustrate that the RPC receive multiple inflow branches (A, B)
and outflow branches (C, D) from the SCP. Schematic illustrations of each pattern are also provided.
of the inner plexiform layer and INL, it traverses a region of high neuronal synaptic activity where the osmolality and biochemical environment is susceptible to rapid fluctuations.  

Similar to the vasa recta, the ICP demonstrates a looped configuration. We postulate that pericyte-mediated mechanisms control blood flow within the ICP that, in turn, serves to buffer the extracellular environment during different states of activity, such as photopic and scotopic vision. For example, by increasing blood flow in the ICP, it may be possible to decrease the osmolarity of the retinal interstitium thereby enhancing synaptic transmission and neuronal excitability. In addition to pericytes, this buffering property may be aided by the uneven expression of proteins and enzymes involved in transcellular endothelial transport across the ICP, such as the Rho family of GTPases, the claudin family of proteins, and a broad range of cytokines, including IL-1, IL-4, IL-10, and IL-10.  

We emphasize that the above hypotheses were not specifically investigated in the present study and require further research for clarification. Other reasons that may explain the variations in capillary circulation morphology between retinal layers may relate to their distinct role in heat exchange, retinal thermal regulation, and maintaining immune privilege. These are other fields that to date have been poorly studied in the retina and require further investigation.

**Figure 11.** Inflow and outflow connections between the ICP/DCPs and the SCP occur at multiple branch points. Three-dimensional constructions of a confocal volume, presented at 2 different angles, illustrate that the deeper capillary plexuses receive multiple inflow branches (A, B) and outflow branches (C, D) from the SCP. Schematic illustrations of each pattern are also provided.
In this study, we found evidence to suggest that regional specializations of retinal capillary bed morphology serve to fine-tune and redirect blood to regions of elevated energy demand. For instance, the RPC were characterized by Y and H branching patterns as well as closed and open loop vascular configurations. We speculate that such topologic patterns allow transient nonperfusion or reperfusion of selective capillary beds, via pericyte-mediated mechanisms, in response to momentary changes in metabolic demand. There remains conjecture regarding the predominant site in the vascular tree where autoregulation occurs. In the brain, arterioles play a key role in regulating blood flow. Vanzetta and colleagues investigated activity-evoked cerebral blood volume changes by using high-resolution optical imaging and showed that the active vascular response is initiated and greatest at the arteriolar level. Kornfield and Newman performed a similar study in the retina by using functional hyperemia as an experimental paradigm. They showed that flickering light evokes large and rapid arteriolar dilation but smaller and slower dilation of downstream venules and capillaries and concluded that functional hyperemia in the retina is driven principally by the arterioles. They also showed that blood flow in the trilaminar vascular network of the rat retina is differentially regulated and reported that flicker stimulation evoked a significantly greater dilation of capillaries in the intermediate layer than the superficial or deep layers. Taken together, spatial precision of oxygen and nutrient delivery within the retina appear to be achieved through micro-compartmentation of autoregulatory mechanisms. The present study did not investigate the dynamic or functional properties

**Figure 12.** Type 1 outflow pattern of capillaries in the SCP. This was characterized by a radial peripapillary capillary in the same plane of the SCP connecting with a single capillary segment of the SCP. Three-dimensional constructions of a confocal volume, presented at 3 different angles (A–C), highlight the outflow pattern. The branch point of interest is denoted by the Inset on Panel A. Trajectories of relevant capillary segments are highlighted by false-colored overlays. A schematic illustration of this branching pattern is provided in the right panel. Pre-capillary arterioles are marked on each image.
of the retinal circulation; however, the organization of the peripapillary vasculature in series and parallel suggests that blood flow in the human retina may be regulated according to the metabolic demands of the different neuronal layers in the retina.

Our results support the seminal work by Paul Henkind in 1967 who found that the RPC are not derived from arterioles within the optic disk but rather from arterioles that supply the peripapillary regions. Toussaint and colleagues speculated that the RPC might be supplied by the SCP, but it was the recent work using pig eyes, by Fouquet et al., that showed that the SCP is the exclusive recipient of blood flow from retinal arterioles. Our study was able to confirm similar findings in human eyes. The unique organization of arterioles, venules, and capillaries in the retina may be explained by the sequence of vascular development that occurs during embryogenesis and the postnatal period. Henkind et al. examined the growth and development of retinal vessels in kittens and found that the RPC and deep capillary networks developed directly from, and after, the SCP. Such a model implicates a high degree of connectivity between the SCP and other retinal capillary beds.

In this study, we found a large number of feeder vessels (62.8%) connecting the SCP to the RPC. Presumably, the greater flow of blood and nutrients to the RPC might be one reason why the RPC are relatively preserved in the early stages of microvascular diseases, such as diabetic retinopathy where the DCP is preferentially affected. Similarly, we found a greater number of drainer vessels (41.9%) connecting the RPC to the SCP. This drainage pattern may have relevance for understanding the pathogenesis of retinal vein occlusion where the deep circulation is also thought to be affected early and more severely. This may be due to the relative paucity of outflow channels within the deeper networks, resulting in greater back-pressure and hydrostatic pressure-mediated neural injury. We emphasize that these hypotheses need to be clarified with further investigations.

Recently, Garrity et al. proposed a geometric model for understanding the organization of the retinal microcirculation by using optical coherence tomography angiography (OCTA) data. Similar to our findings, Garrity et al. proposed that the SCP and ICP were not distinct capillary units with a separate arterial supply and venous drainage system. Similar to our report, they also proposed that anastomotic capillary bridges allowed communication between the SCP, ICP, and DCP.

**Figure 13.** Type 2 outflow pattern of capillaries in the SCP. This was characterized by a capillary of the ICP or DCP and a capillary in the same plane of the SCP connecting with a single capillary segment of the SCP. Three-dimensional constructions of a confocal volume, presented at three different angles (A–C), highlight the outflow pattern. The branch point of interest is denoted by the inset on panel A. Trajectories of relevant capillary segments are highlighted by false-colored overlays. A schematic illustration of this branching pattern is provided in the right panel. Precapillary arterioles are marked on each image.
However, they suggested that the DCP serves as the primary site of venous outflow for the entire retinal microvasculature, whereas our work did not support this finding. There are two important methodologic differences between our work and that of Garrity et al.45: (1) we visualized perfusion-labeled histologic data by using confocal scanning laser microscopy, and (2) our work concentrated on the peripapillary regions and did not include the macula. The landmark paper of the macaque retina performed by Shimizu 46 was consistent with our histologic findings, as it also did not report the vortex nature of the deep capillary circulation in the peripapillary region as proposed by Garrity et al.45 These differences may relate to the depiction of the deep retinal circulation, as seen on OCTA versus postmortem histology. It may also reflect variations in the organization of the deep capillary circulation between the macula, peripapillary region, and other retinal eccentricities. Further work will be required to reconcile these differences.

A publication by Shimizu 46 in 1978 provided a detailed account of the organization of the monkey retinal microcirculation by using corrosion casting and scanning electron microscopy. Similar to our report, Shimizu 46 reported that the peripapillary circulation is laminated and organized into distinct capillary plexuses. However, unlike our work, Shimizu 46 reported directed communications between capillaries of the deep circulation and the retinal veins. There are several explanations that may explain these differences, including inherent differences in the morphology of the retinal circulation between monkeys and humans. For example, the central retinal artery in the monkey branches into five to six
major branches at the optic nerve head instead of three to four in the human.47 Additionally, the terminal capillaries bordering the foveal avascular zone in humans lie at the level of the GCL, whereas it lies at the level of the INL in monkeys.2 There were also differences in tissue preparation and imaging. Shimizu’s observations were based on scanning electron microscopic images of retinal plastic casts.46 Although this is an excellent technique for preserving retinal vascular morphology, post-mortem digestion of nonvascular tissue following polymerization of the casting compounds results in the loss of spatial detail due to pressure artefacts and may alter the three-dimensional relationships between networks. Therefore, it can be difficult to precisely colocalize vascular structures to distinct retinal layers by using this technique.48 In our study, we did not perform postperfusion digestion of tissue and were able to colocalize the position of vessels to different retinal layers by using nuclear labeling (Fig. 1). Using Imaris Image Analysis software (Bitplane), we were also able to rotate three-dimensional images of the microcirculation in the x-, y-, and z-axes and use optical sectioning to precisely study the connections between capillaries, arteries, and veins and carry out sectioning of the three-dimensional volume when necessary.

The findings from our study will significantly aid our ability to interpret two- and three-dimensional data acquired through OCTA data in the clinical setting. Volume rendering OCTA is emerging as a powerful technique for studying the morphometric characteristics of the retinal circulation as well as studying the association between vascular changes and retinal structural alterations, such as the development of cystoid edema.49 Application of such techniques to study microvascular organization and the preferential involvement of retinal capillary beds in certain pathologies may identify a novel method for stratifying disease progression.

**FIGURE 15.** Type 4 outflow pattern of capillaries in the SCP. This was characterized by a branch of the radial peripapillary capillary plexus, a capillary of the ICP or DCP and a capillary in the same plane of the SCP connecting with a single capillary segment of the SCP. Three-dimensional constructions of a confocal volume, presented at three different angles (A–C), highlight the outflow pattern. The branch point of interest is denoted by the inset on panel A. Trajectories of relevant capillary segments are highlighted by false-colored overlays. A schematic illustration of this branching pattern is provided in the right panel. Precapillary arterioles are marked on each image.
We acknowledge several limitations of this study, namely the limited sample size. As there is marked variation in normal vascular anatomy between individuals, the limited sample size may have precluded identification of less common topologic patterns of capillary organization. Finally, our speculations regarding vascular physiology are solely based on the angioarchitectural (structural) properties of the retina and do not account for the non-Newtonian rheologic characteristics of the retinal circulation.

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