Quantitative Study of the Macular Microvasculature in Human Donor Eyes

Paula K. Yu,1,2 Zaid Mammo,3 Chandrakumar Balaratnasingam,1,2,4 and Dao-Yi Yu1,2

1Centre for Ophthalmology and Visual Science, The University of Western Australia, Perth, Australia
2Lions Eye Institute, The University of Western Australia, Perth, Australia
3Department of Ophthalmology and Visual Sciences, University of British Columbia, Vancouver, British Columbia, Canada
4Sir Charles Gairdner Hospital, Perth, Australia

Correspondence: Dao-Yi Yu, Lions Eye Institute, Centre for Ophthalmology and Visual Science, The University of Western Australia, Nedlands, WA 6009; dyyu@lei.org.au.
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PURPOSE. To precisely quantify the macular microvasculature density using microperfusion and labeling techniques in human donor eyes. Such information may be useful in understanding the role of the macular microvasculature in coping with the metabolic requirements of the neurons in this densely packed region, and provide a reference point for clinical studies using recently developed optical imaging techniques.

METHODS. The macular microvasculature was perfusion-labeled in 18 human donor eyes and optical stacks collected from regions superior, temporal, inferior, and nasal to the foveola using confocal microscopy. The optical slices were separated into the deep macula vascular layer (DL) and the superficial layer (SL) in which all the vessels superficial to the deep macular vessel layer were included. The DL and SL images were analyzed and vessel density measured according to their orientation from the foveola and in foveal and parafoveal regions. Vessel densities were compared across regions and age groups.

RESULTS. Both the SL and DL showed an increase in vessel density with increasing eccentricity from the foveal to parafoveal regions. Vessel density was found to rank in the order of inferior > superior > temporal > nasal in both SL and DL layers. The SL vascular density was approximately 31%, whereas DL was approximately 17%. The DL was planar in nature and density not affected by age. Age-related increase in vessel density was observed in the SL.

CONCLUSIONS. Microperfusion and labeling techniques in combination with confocal microscopy has enabled collection of reliable data on vascular density in the macula region. Regional differences may reflect well-matched vascular supply and neuronal demands. Age-related changes might indicate the importance of stable blood supply for the human macula.

Keywords: retinal vasculature, macula, human

The human macula or macula lutea is a highly specialized region responsible for almost the whole of our visual acuity. It is a region with the highest concentration of cones and retinal ganglion cells. Knowledge of the coupling between the macular vasculature and dense macular neurons is important. The relative sparsity of vasculature in the macular region is thought to maximize access for photons to reach the photoreceptors; however, this seemingly paradoxical demand and supply mismatch may also present vulnerability in disease situations in which retinal blood supply is affected.

Recent advances in noninvasive imaging technologies have opened opportunities to investigate the ocular vasculature in greater detail. Several studies have attempted to quantitatively compare the vascular density at the macular region in healthy, aged, and diseased eyes. Although the measurements are highly repeatable and reproducible within studies, large discrepancies in vessel density exist between studies. The morphology and density of retinal microvasculature have previously been studied using various techniques, including injection of India ink, latex, benzidine peroxidase staining, infusion of silver nitrate, periodic acid-Schiff technique, casting, ADPase enzyme histochemistry, and fluorescein angiography. More recently, optical coherence tomography angiography (OCTA) and adaptive optics have been used for quantifying macular microvasculature clinically as a noninvasive technique. Various algorithms have been used to obtain vascular density data. A study to compare regional differences in vascular density around the macular region also has been conducted. However, comparison of data from these studies found wide-ranging variation from a minimum of 27.6% to more than 90.0% in the macular region. Factors contributing to the variation in vascular density are many fold and include vessel-shadowing artifacts, technical limitations in lateral resolution at 6 μm, and differences in the segmentation applied for inclusion of retinal layers and thickness in obtaining density measurements. Several studies have since addressed the confounding issue of segmentation using layering information derived from several confocal studies on the human retina, as well as the study by Snodderly and Weinhaus on monkey macular capillary layers. Later studies have that compiled histological knowledge to segmentation procedures have provided lower measurements for vascular density. Large variation in recent data casts an obvious doubt on the accuracy of vascular density measurements. As stated by Shahlaee et al., there is currently no “gold standard” against which they can validate their quantification data.
We have used microcannulation, perfusion, and labeling techniques to study the entire retinal vasculature \(^{27-30}\) including the macular microvasculature. \(^{27,31}\) In combination with confocal microscopy, we were able to visualize endothelial cells of macular microvasculature and precisely and accurately quantify the vascular density including the deepest planar capillary in the macular region. For the present study, we quantified the vessel density of the superficial layer (SL) and the deep layer for the foveal and parafoveal regions according to the definition by Hogan et al.,\(^{32}\) as well as for the superior, inferior, nasal, and temporal regions of the macula. We also reported the regional differences of vascular density in young and aged groups.

**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**

Eighteen eyes obtained from 16 donors were used for this study. All eyes were obtained from the Lions Eye Bank of Western Australia following valid consent for use of such tissue for research purposes. Fifteen eyes were received after the removal of corneal buttons for transplantation and three eyes were received intact. None of the donors had a known history of diabetes, glaucoma, ischemic optic neuropathy, or central retinal artery (CRA) or central retinal vein occlusion. The demographic data, cause of death, and postmortem time to eye perfusion are listed in Table 1.

**Microcannulation and Perfusion Labeling**

All 18 eyes were cannulated at the CRA and perfusion-labeled. With the exception of donor eye R, all eyes were labeled with Phalloidin conjugated to Alexa Fluor 546 (A22283; Invitrogen, Carlsbad, CA, USA) or Tetramethylrhodamine (TRITC) (P1951; Sigma-Aldrich Pty Ltd., St. Louis, MO, USA). Donor eye R was perfusion-labeled using Lectin TRITC from Triticum vulgaris (L5266; Sigma-Aldrich). Perfusion commenced on average 14.5 ± 1.40 hours postmortem.

Detail protocol of the microcannulation and perfusion technique was published previously. \(^{27}\) Briefly, the CRA was cannulated using a glass micropipette with a tip diameter of approximately 120 to 160 μm and a series of solutions perfused through at a rate of 50 μL/min. The solutions were perfused in the following order: Ringer’s solution with 1% BSA (20 minutes), 4% paraformaldehyde in 0.1M phosphate buffer (30 minutes), 0.1M phosphate wash buffer (15 minutes), 0.1% Triton X-100 in 0.1M phosphate buffer (5 to 7 minutes), 0.1M phosphate wash buffer (15 minutes), 30 U Phalloidin in 0.1M phosphate buffer (three boluses over the course of 1.5 hours), 0.1M phosphate buffer (30 minutes). Nuclei counterstain in the form of bis-Benzamide H (B2261; Sigma-Aldrich) or YOPRO-1 (Y3603, Invitrogen) usually accompanied the Phalloidin perfusion, although information from the nuclei channel was not used in this study.

**Retinal Flat Mounting and Confocal Imaging**

After perfusion labeling, the eye was left immure fixed overnight in 4% paraformaldehyde (in 0.1M phosphate buffer). The retina was dissection out the next day and flat-mounted onto glass slides using glycerol. The correct orientation of the retina for imaging was determined via the naming convention of the eye bank for left and right eyes, by visual confirmation of external landmarks from remnants of ocular muscle insertion points. \(^{35}\) The inferior location of the CRA as well the retinal vascular distribution pattern upon flat mounting.

Confocal images were collected from the macula region on the Nikon C1 system using ×10 Plan Apo objective lens. As far as possible, image stacks were collected at 1.15-μm step size in the four “quadrants” of the macula; namely, superior, inferior, temporal, and nasal (direction toward the optic disk) in relation to the foveola (Fig. 1). Confocal images were collected at 1024 × 1024 pixel resolution for each image field measuring 1270 × 1270 μm (1 pixel = 1.24 μm). Each image stack is collected to include the uppermost (vitread) and outermost (scleral) retinal vessel that can be detected.

**Image Processing**

Each image stack was segregated into superficial and deep layers (Figs. 2, 5). The deepest laminar layer imaged was defined as the outermost layer of vessel included in the image stack. The SLs included all vessels above the deep layer.

Optical sections from each layer were projected as a maximum-intensity two-dimensional image. Two-dimensional images from different locations of each specimen were aligned using Adobe Illustrator CS4 (Adobe Systems Inc., San Jose, CA, USA) such that a single composite image of vascular pattern of each macula was made. The resultant image composite enabled distance calibration and the subsequent placement of a calibrated grid onto the image composite for definition of measurement zones. The definitions of Hogan et al.\(^{32}\) for foveola (350-μm diameter centered on fovea), fovea (750-μm annulus surrounding fovea), and parafovea (500-μm annulus surrounding fovea) regions was applied to each region.

Macula vessels were manually traced for each image using the GNU Image Manipulation Program (GIMP version 2.8.14; The GIMP Development Team, Free Software Foundation, Boston, MA, USA). The SLs and deep layer were put side-by-side to compare for projection artifacts and avoid double tracing. The traced image was used for measuring percentage vessel occupation per region using Image Pro Plus (version 7.1; Media Cybernetics, Rockville, MD, USA). The expression of vessel density is in relation to a two-dimensional area. Vessel density
RESULTS

Microcannulation and perfusion technique enabled labeling of the entire macula microvasculature within the structure of the neuro-retina. This is evidenced from the presence of continuity in vessel connection throughout the optic image stacks (Fig. 1). The average age of the 18 donors was 47.1 ± 5.12 years (range 22 to 79) and was further separated into two groups. Nine donor eyes from eight younger donors (average age of 27.3 ± 1.94 years, range 22–39, n = 8) as one group, and nine donor eyes from eight older donors with (average age of 66.9 ± 2.15 years, range 60–79, n = 8) as another group.

Planar Nature of Deep Macula Capillaries

The deep capillary layer in the macula region was found to be very laminar in all quadrants and occupies a retinal thickness of 9.7 ± 5.94 μm (n = 58) in the fixed whole-mounted retina. Figure 2B and Figure 3 show the laminar nature of the deep capillary layer bordering the scleral edge of the inner nuclear layer. There was no significant difference in the deep capillary layer occupied retinal thickness (P = 0.923) with age.

Deep Layer (DL) Vessel Density

Vessel density of the deep macular vascular layer was consistently at approximately 17% when analyzed as pooled data or separately according to age to include both the foveal and parafoveal regions (Fig. 4). The parafoveal region has a significantly higher vessel density than the foveal band (P < 0.001), which is consistent across both age groups. Comparison by quadrant found nasal quadrant to be significantly sparser than the superior quadrant (P = 0.039), whereas measurements between other quadrants are comparable (see Fig. 3 DL). Table 2 lists the averaged measurements for each of the study regions. Table 3 lists the pooled average for each region grouped by donor’s age.

SL Planar Stack Vessel Density

The SL included all vessel plexuses above the plane of the DL and was found to be significantly denser than the DL. Overall, the pooled vessel densities (from all quadrants and regions) of the SL were approximately 31.5% (Table 4) and ranged between 29% and 33% across the four quadrants. The parafoveal region SL was consistently denser than the foveal SL (P < 0.001), and true for both age groups (Table 3). Three-way ANOVA analysis found the superior and inferior quadrants to have significantly higher percentage vessel occupation than the nasal and temporal quadrants (P < 0.05). Separate analysis by age groups did not find such significant difference between quadrants in the older eyes, but identified the younger donor eyes to be the main contributor to the interquadrant differences.

Comparison across age groups found older eyes have significantly denser SL (Fig. 5) than the younger donor eyes in all quadrants (P < 0.001) and in both the foveal and parafoveal regions (P < 0.001).

Both layers showed a similar trend in density measurements between quadrants where inferior > superior > temporal > nasal and was consistent across the age groups.

SL Vessel Diameter

The vessel diameters were measured for different orders of vessels (A4, A3, A2, A1, capillary, V1, V2, V3, and V4) and analyzed according to age and presented in Table 5. Significantly wider vessels were noted in the eyes from older donors, in particular for vessel orders A4, A3, A2, capillaries, V1, and V2.

DISCUSSION

The major findings of this study are as follows: (1) the DL occupies 17% of the plane and increases with eccentricity from...
the foveola, (2) the DL density is unchanged with age, (3) the SL projected layer density is approximately 30% and the density is found to be significantly higher in the older age group, and (4) regional-specific differences are present in vascular density in the order of inferior > superior > temporal > nasal.

These qualitative data of the vascular density in this study provides precise information of the macular microvascular network that could be a reference point for recent clinical quantitative study on macular microvasculature using OCTA technique. OCTA is a valuable label-free tool for imaging the

FIGURE 2. Superficial and deep layers. Confocal images show the SL and deep layers of the temporal quadrant from donor C (32 years old) imaged using Plan Apo ×10 lens. (A) The SL is a projected image of all optical sections above the deep layer. The optical sections have been separated into three layers and color-coded according to their location in the retina to indicate the relative position of the vascular layers. The top one-sixth of the superficial retinal thickness is colored blue, the next one-third of the SL retinal thickness was colored green, and the rest of the SLs were colored red. (B) shows the deep layer of the vessel as a separate image.

FIGURE 3. Four regions of superficial and deep layers imaged at a higher magnification using Plan Apo ×10 lens. Four sets of images showing the vascular pattern of SL and DL in the four quadrants from the macula of donor E. Each image measures 1270 × 595 µm in area and includes part of the foveolar region.
retinal circulation; however, there is a wide-ranging variation from the quantitative data reported.\textsuperscript{3,4,6} In this study, we applied the micromanipulation, perfusion-labeling technique in combination with confocal imaging of retinal microvasculature in the flat-mounted specimens from human donor eyes.\textsuperscript{27-30} Such combination of techniques can reliably label the entire retinal vasculature and each endothelial cell. In this study, with the resolution of 1.15 \(\mu\)m step size in image stacks and 1.24 \(\mu\)m per pixel on screen resolution, we were able to visualize endothelial cells of macular microvasculature to quantify accurately the vascular density, including the deepest planar capillary in the macular region.

We have previously reported the detailed topographic distribution of macular microvasculature and intracellular structure of endothelium. The macular vasculature supporting this highly specialized region has a specific topography\textsuperscript{31} with almost nine pairs of alternating retinal arteries and veins radially arranged around the avascular foveola. The macular arteries give off numerous branches in the parafoveal and foveal regions of the macula (average of 11.6) with some branches diving deep at a right angle to supply the deeper macula,\textsuperscript{31} whereas others continue to branch more superficially\textsuperscript{19} before finally joining the retinal venous plexus in the deeper macular layers.\textsuperscript{14,31} Such topographic distribution is consistent with the current report of SL and DL densities being higher in the parafoveal region than the foveal region (Tables 2, 4). The inclusion of the peripheral portion of the avascular foveola in the foveal region would have contributed somewhat to the lower density observed in this region. The pooled average of DL density at 17% is similar to that reported for perifovea\textsuperscript{23} previously and comparable with the peripheral retina at 16.12\textsuperscript{24}. We identified an increase in vessel density with increasing eccentricity between the foveola and parafoveal region of 14% to 20%, which corresponds very well with the eccentric increase in rods\textsuperscript{1} and microglia\textsuperscript{54} density, suggesting a dependency relationship of the rod photoreceptors on the DL. The density of macular DL also was found to be very stable across the age groups. Whereas DL vessel density is reported to decrease in the peripheral retina but increase in the perifoveal region with vascular comorbidity,\textsuperscript{29,35} further comorbidity studies will be required to determine how the macular DL density may be altered in different disease conditions.

The DL resides at the outer border of the inner nuclear layer, on par with horizontal cell layer. Its proximity to the outer plexiform layer (OPL) suggests that DL is essential in meeting the high energy demand of the OPL, which is composed of synapses between the cone-rich photoreceptor layer, and the bipolar and horizontal cell in this specific region. Previous study by Gao and Hollyfield\textsuperscript{27} on ageing of the retina found the densities of foveal cones to be stable throughout the second to ninth decades. Similar stability is demonstrated by the lack of change in vessel density in the DL between the two age groups in the current and previous studies,\textsuperscript{38} supporting the presence of stability in the deep macula layer.

The layering of macular microvasculature is more complicated due to the slope toward the fovea pit and the avascular foveolar zone. Epoxy sectioning of human retina has identified a shift in the position of the superficial vascular plexus through different anatomic neuronal layers of the retina as the retina thickens with increased eccentricity from the center of the pit.\textsuperscript{39} This has created some difficulties in the specific localization of the various layers in the superficial vascular plexus, because the same planar layer would have changed position in relation to the anatomic neuronal layers. The projection of the multilayer vascular network into the SL has resulted in a much higher vascular density than the DL. Interestingly, a significant increase in SL vascular density has been identified in the older donor eyes. SL density was found to be significantly higher in the older donor eyes, independent of the DL vascular density. This was an unexpected finding, as quite a few studies have identified an age-related decrease in retinal vasculature area,\textsuperscript{40} ganglion cell layer

### Table 2. Pooled Vessel Density of Deep Macula Layer (DL)

<table>
<thead>
<tr>
<th>Pooled Vessel Density</th>
<th>Percentage of Area Occupation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From All Eyes</td>
</tr>
<tr>
<td></td>
<td>Foveal</td>
</tr>
<tr>
<td>Foveal + parafoveal regions DL</td>
<td>17.4 ± 5.14 (140)</td>
</tr>
<tr>
<td>Superior quadrant DL</td>
<td>18.7 ± 4.89 (34)</td>
</tr>
<tr>
<td>Temporal quadrant DL</td>
<td>17.1 ± 5.28 (36)</td>
</tr>
<tr>
<td>Inferior quadrant DL</td>
<td>17.7 ± 4.88 (36)</td>
</tr>
<tr>
<td>Nasal quadrant DL</td>
<td>15.8 ± 5.27 (34)</td>
</tr>
<tr>
<td>Foveal region DL</td>
<td>14.2 ± 4.01 (70)</td>
</tr>
<tr>
<td>Parafoveal region DL</td>
<td>20.5 ± 4.17 (70)</td>
</tr>
</tbody>
</table>

Vessel density is expressed as percentage ± SD; n value in parentheses indicates the number of measurements included in the calculation of mean value.

### Table 3. Percentage Area of Vessel Occupation Grouped by Age and Region

<table>
<thead>
<tr>
<th>Pooled Vessel Density</th>
<th>From All Eyes</th>
<th>Foveal</th>
<th>Parafoveal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Younger DL</td>
<td>14.6 ± 4.61 (35)</td>
<td>20.0 ± 4.51 (35)</td>
<td></td>
</tr>
<tr>
<td>Older DL</td>
<td>14.0 ± 3.32 (35)</td>
<td>21.0 ± 3.68 (35)</td>
<td></td>
</tr>
<tr>
<td>Younger SL</td>
<td>27.0 ± 3.59 (35)</td>
<td>31.1 ± 4.88 (35)</td>
<td></td>
</tr>
<tr>
<td>Older SL</td>
<td>30.2 ± 4.54 (36)</td>
<td>36.6 ± 4.09 (35)</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of measurements included in the calculation of mean value.

### Table 4. Pooled Vessel Density of Macula SL

<table>
<thead>
<tr>
<th>Pooled Vessel Density</th>
<th>Percentage of Area Occupation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From All Eyes</td>
</tr>
<tr>
<td>Foveal + parafoveal regions SL</td>
<td>31.3 ± 5.52 (141)</td>
</tr>
<tr>
<td>Superior quadrant SL</td>
<td>32.7 ± 5.26 (34)</td>
</tr>
<tr>
<td>Temporal quadrant SL</td>
<td>30.1 ± 4.80 (36)</td>
</tr>
<tr>
<td>Inferior quadrant SL</td>
<td>33.3 ± 5.62 (36)</td>
</tr>
<tr>
<td>Nasal quadrant SL</td>
<td>29.3 ± 5.52 (35)</td>
</tr>
<tr>
<td>Foveal region SL</td>
<td>28.7 ± 4.42 (71)</td>
</tr>
<tr>
<td>Parafoveal region SL</td>
<td>35.9 ± 5.30 (70)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of measurements included in the calculation of mean value.

### Table 5. Diameters of Retinal Microvessels Grouped by Vessel Order and Age

<table>
<thead>
<tr>
<th>Vessel Order</th>
<th>Young</th>
<th>Old</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>40.7 ± 1.42 (62)</td>
<td>39.8 ± 0.89 (78)</td>
<td>0.009</td>
</tr>
<tr>
<td>A3</td>
<td>20.0 ± 0.43 (141)</td>
<td>21.7 ± 0.31 (128)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A2</td>
<td>14.7 ± 0.13 (240)</td>
<td>16.1 ± 0.12 (222)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A1</td>
<td>11.6 ± 0.11 (301)</td>
<td>12.0 ± 0.10 (317)</td>
<td>0.951</td>
</tr>
<tr>
<td>Capillaries</td>
<td>8.1 ± 0.09 (370)</td>
<td>8.2 ± 0.07 (426)</td>
<td>0.043</td>
</tr>
<tr>
<td>V1</td>
<td>11.0 ± 0.21 (196)</td>
<td>11.7 ± 0.12 (222)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>V2</td>
<td>14.9 ± 0.20 (146)</td>
<td>15.8 ± 0.13 (153)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>V3</td>
<td>25.1 ± 0.93 (112)</td>
<td>22.0 ± 0.34 (109)</td>
<td>0.225</td>
</tr>
<tr>
<td>V4</td>
<td>43.6 ± 1.22 (54)</td>
<td>41.4 ± 1.28 (67)</td>
<td>0.146</td>
</tr>
</tbody>
</table>

Measurements are in microns. Numbers in parentheses indicate the number of vessels measured. \(P < 0.05\) is considered significant.
thickness, reduction in ganglion cell density, and rod photoreceptor cell and rod bipolar cell density with aging. The decrease in neuronal cell numbers would likely be associated with reduction in metabolic demands and possibly a reduction in vessel density as indicated by previous and recent reports. Even in subjects with vascular comorbidity, a significant decrease was reported for the SLs of the peripheral retina and no density change was reported for any of the layers in the perifoveal region. However, an increase in SL density was observed in the older donor macula. Our attention was therefore drawn to the possible effect from comorbidity, as most donors in the older age group died from cardiac-related events. It is known that age is an independent factor that accompanies an increase in retinal arteriole wall thickness and wall cross-sectional area. Comparison of vessel diameters in different order of vessels in the SL was subsequently made across the two age groups. The results confirmed the presence of wider

**Figure 4.** Deep layer of donor R labeled by Lectin TRITC. This is a composite image showing the deep laminar capillary plexus of donor eye R, also imaged using a Plan Apo ×10 lens. The average thickness of this deep capillary layer is 8.6 ± 1.48 μm. The vascular density of the deep layer in older donors is comparable to those in younger donors (Figs. 2, 3).

**Figure 5.** SLs of donor R (62 years old) labeled using Lectin TRITC. This is a composite image compiled from higher magnification (Plan Apo ×10 lens) image stacks showing the SLs of the macular region. Each vessel can be clearly discerned at this magnification where 1 pixel = 1.24 μm in resolution. There is an obvious increase in vessel density compared with those from younger donors (Figs. 1–5).
microvessels in the SL of older donor eyes, which could contribute significantly to the increase in SL vessel occupation measurements. Similarly, elevated blood pressure is also associated with increase in retinal arteriole thickness and wall cross-sectional area, with systemic hypertension previously reported to induce pericyte changes leading to wall thickening at the capillary level. It is possible that similar effects of aging and systemic cardiovascular changes are present in the macular region microvasculature, contributing to the higher vessel density reported in the older age group. Further study comparing data from normotensive donors against those with vascular comorbidities may help to elucidate this point.

A recent study on monkey eyes identified an increase in microglia density with age in the macular region. As the microglia are known to have a tiled distribution in the inner retina with processes concentrated in the inner plexiform layer and OPL, their increased presence in aged eyes could have contributed to the increased metabolic demands of the inner retina, potentially inducing increased vascular growth and inflammatory responses. More detailed study on the age-related change in microglia and metabolic demand of the human inner macular will be needed to clarify this point.

Regional difference in vascular density has been found in this study in the order of inferior > superior > temporal > nasal. The presence of major retinal arteries and their branches in the superior and inferior margin of the macula would most likely contribute to the higher measurements of vascular density in the SL. The nasal margin of the macula is interesting, as it had been demonstrated to have a visual streak-like configuration due to the presence of horizontal extension in high neuron density from the foveal eccentricity. Such cells included cones and catecholaminergic-positive amacrine cells. Yet the nasal rim has the lowest vascular density in the foveal and parafoveal regions. Perhaps the richness of perifoveal vascular plexus and the proximity of this region to the larger retinal arteries at the optic disc also helps to support the metabolic needs of this region.

Some recent publications have applied automated segmentation to OCTA data to separate the macular microvessel layers into two layers: superficial capillary plexus and deep capillary plexus for quantitation of vascular density, with most reporting approximately 50%. Two recent OCTA studies have applied the laminar vascular layer structure learned from study of peripheral and perifoveal retina to manually segment out the deep vascular layer in the macular region for qualitative studies. However, shadowing from more superficially located vessels onto the segmented image of the deepest plexus confounds accurate imaging and measurements. This study forms part of the confocal study series to obtain reliable quantitative data to further study of macular vessels. We confirm that the deep macular vessel layer is highly laminar in nature, similar to that reported for the peripheral and perifoveal retina.

SL density was found to be approximately 34% in the parafoveal region. This is significantly lower than the vascular density reported based on OCTA images at 46% to 58%, suggesting an overestimation using OCTA images. The higher percentage could be due to lower lateral resolution, motion artifacts, and inclusion of the deep layer plexus.

Regional-specific differences have also been reported based on OCTA data and matched a similar trend in density difference in which inferior/superior > nasal/temporal, although the reported density is approximately 50% to 60%, double that of our measurements. This agreement in trend provided some assurance to the relative precision of the OCTA-based data, although the accuracy is not comparable to the histological data.

Obvious differences between histology and OCTA technology contributed to the differing results. First, the resolution of OCTA is far coarser than our confocal system for images collected on the ×10 objective lens. The resolution of the confocal technique used in the current study is at least 7- to 8-fold better than the OCTA-generated images, and is likely the main factor for the much lower density measurements. Second, shadowing effect from vessels located more anteriorly is known to overlay signal from vessels lying deeper to them. On the other hand, confocal technique can eliminate the stray light and provide better image quality, thus largely eliminating the interference from vessels overlying the deeper ones. Third, the OCTA technique relies on optical clarity of the structures anterior to the part of the tissue being imaged; namely, that includes the cornea, lens, and vitreous in front of the retina. Cloudiness in any of these structures will greatly affect the quality of the images obtained from OCTA, although all of these structures would have been removed from retina prepared for confocal microscopy. Fourth, successful OCTA imaging relies on continuous flow in the microvasculature. As we know, blood flow in the microvasculature is not always continuous, which has been reported in the heart and other organs. Recently, OCTA images captured from the same macula at different times also showed varied intensities of the same microvessels (Yu D-Y, unpublished data, 2017), which may indicate that blood flow in the macular microvasculature is not always continuous. The mechanisms controlling flow within the macula microvasculature are currently unclear and awaiting further investigation. In comparison, confocal microscopy uses an intravascularly perfused probe that is taken up by factor in all endothelial cells. Because the probe is perfused under positive pressure through the distribution network of the CRA, the entire microvasculature can be labeled for more accurate representation of the complete microvasculature. Fifth, OCTA is confounded by saccadic motions from live subjects, whereas confocal imaging is on fixed tissue that is mounted on glass slides. The motions experienced during OCTA will also increase noise level in the image, making it difficult to delineate true signal from noise. Finally, there is no comparable quality of OCTA image that allows accurate quantitative study on the deep vascular layer. Hence, although the development of OCTA has contributed significantly to the noninvasive observation of intraocular structures, further advancements are needed for more accurate and true imaging.

The results of this study may serve as a reference to which improvement in OCTA technologies may compare. Further quantitative studies on the segregation of the SLs will improve our understanding of the macular vascular structure and contribute to the building of a more meaningful segmentation algorithm.

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