Segmentation of the Four-Layered Retinal Vasculature Using High-Resolution Optical Coherence Tomography Angiography Reveals the Microcirculation Unit

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Purpose. To differentiate the four layers of the retinal vessel network in the human macula and examine their morphologic features using high-resolution optical coherence tomography angiography (HR-OCTA).

Methods. Macular areas measuring 464 × 464 pixels of 10 right eyes of 10 healthy subjects without ocular disease were scanned 10 times using a HR-OCTA device. Averaged OCTA images were created. Based on clear decorrelation signals, four vascular slabs were segmented, comprising one each in the retinal nerve fiber layer (RNFL), ganglion cell layer (GCL), and top and bottom of the inner nuclear layer (INL). Qualitative features and quantitative measurements in each slab were compared with those in conventionally segmented slabs.

Results. HR-OCTA isolated four layers of vascular plexuses in the macula that followed the corresponding anatomic layers. Segmentations for the RNFL revealed that radial peripapillary capillaries (RPCs) extended to the central macular area. The RPCs followed relatively straight and long paths, with few apparent feed points and anastomoses. The GCL slab enhanced visualization of the capillary-free zones around the arteries and arterioles and helped to differentiate arterial and venous systems. The arterioles and venules were linked by capillaries that were arranged in a mesh-like fashion, with multiple arteriolar feed points and anastomoses. Vascular plexuses in the top and bottom of the INL consisted of capillaries in a vortex arrangement. The center of these vortex arrangements was consistent with the venules in the GCL.

Conclusions. HR-OCTA can differentiate the four layers of vascular plexuses in the human macula and elucidate their angiographic features.

Keywords: high-resolution optical coherence tomography angiography, ACV unit, retinal microcirculation unit, four-layered retinal vasculature

In living human eyes, neuronal and glial cells in the inner retina transmit signals from photoreceptors in the outer retina.1 These cells are nourished by retinal vessel plexuses parallel to the retinal plane, each of which is bridged by perforator vessels perpendicular to this plane.1 A previous histologic investigation reported that the peripapillary area contained the most retinal vessels.2 The capillaries in each vessel plexus are described as radial peripapillary, superficial, intermediate, or deep.2,3

Optical coherence tomography angiography (OCTA) shows the retinal capillary plexuses in real time by segmenting the three-dimensional angiographic data into en face slabs of interest.4 This imaging technique has improved our understanding of the pathogenesis of retinal vascular disorders. However, commercially available OCTA devices only show two slabs comprising superficial capillary plexus (SCP) and deep capillary plexus (DCP), with two to three of the four layers of plexuses considered overlapped in each segmented slab.1,5 Moreover, as Spaide and Crucio6 have recently shown, segmentations of vascular plexuses in the central macula are incorrect, even in healthy eyes. Therefore, examination of the retinal vasculature by OCTA with default segmentation might hinder investigation of retinal circulation.

The optical axial depth resolution for common OCTA instruments is 6 to 10 μm.4 However, these values may be inadequate for resolving tiny retinal capillaries. An OCTA system (OCT-HS100; Canon, Tokyo, Japan) with an optical axial resolution of 3 μm is now commercially available.7 The OCT-HS100 can generate high-contrast OCTA with a multiple en face averaging technique. In this study, we used the OCT-HS100 to differentiate the four layers of retinal capillary plexuses in the living human macula and investigated angiographic features of each plexus and relationships between the plexuses.

Methods
OCTA for Macular Area

This prospective study was approved by the Kyoto University Graduate School of Medicine Ethics Committee and conducted in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from each study participant.
Ten healthy subjects (6 men, 4 women) without ocular disease were enrolled. A macular area measuring 4 × 4 mm² (464 × 464 pixels) in the right eye of each subject was scanned 10 times with an OCT-HS100 device. Averaged OCTA images were then created for each subject.

The HS100 (Canon, Inc., Tokyo, Japan) high-resolution spectral-domain (HR-SD) OCT system was used to obtain the study images. The HS100 has an extended bandwidth, superluminescent diode light source with a full width at half-maximum value of 100 nm. The light source comprises two chips that deliver a 3-µm optical axial resolution. In the default setting of the OCT-HS100 device, the lower boundary of the SCP measures 50 µm below the border between the ganglion cell layer (GCL) and inner plexiform layer, which is the upper boundary of the DCP. The lower boundary of the DCP measures 70 µm below the border between the inner plexiform layer and inner nuclear layer (INL).

The optical axial resolution of the OCT-HS100 is 3 µm. The 464 sequentially averaged OCT B-scans for the central macular area displayed clear decorrelation signals representing retinal blood flow in the retinal nerve fiber layer (RNFL), GCL, and top and bottom of the INL, some of which communicated with the GCL and top or bottom of the INL (Supplementary Video S1). The distinct distributions of retinal vessels prompted us to perform novel segmentation such that each of the four layers of retinal vascular plexuses could be isolated separately with the OCT-HS100. The en face angiographic slabs of the first, second, third, and fourth vascular plexuses were created by segmenting the RNFL, GCL, and inner and outer INL components, respectively.

Quantitative Measurements

The vessel density (VD), vessel length density (VLD), vessel diameter index (VDI), and fractal dimension (FD) values measured on OCTA were compared between the default and novel segmentations. For these measurements, a 464 × 464-pixel rectangular box, centered on the fovea, was cropped and binarized with a modified version of a previously reported method. Briefly, after processing with a top-hat filter, the image was duplicated and a different binarization method was performed. One image was processed first by a Hessian filter followed by global thresholding with Huang’s fuzzy thresholding method. The other (duplicate) image was binarized with median local thresholding. Finally, the two separate binarized images were combined to generate the final binarized image, in which only pixels that existed on both binarized images were included.

VD was assessed on the final binarized image and defined as the ratio of the area occupied by the vessels, divided by the total area. After skeletonization of the binarized image, the VLD was evaluated as described previously. The VDI was calculated by dividing the total vessel area in the binarized image by the total vessel length in the skeletonized image. Finally, FD was calculated on the skeletonized image with Fractalx software (ThÉMA, Besançon Cedex, France). The box-counting method was used for calculation. The FD ranges from 0 to 2; images with more complex vessel branching patterns demonstrate a higher FD. Qualitative features, quantitative VD and FD for the vascular slabs segmented with the novel method were compared with those of slabs in the SCP and DCP that were segmented with the default method.

In addition to automated measurement of VD, VLD, VDI, and FD, we measured vessel luminal diameters semiautomatically with OCTA images for each vascular plexus and a previously reported method. Luminal diameters of the arteries, arterioles, capillaries, veins, and venules were averaged from measurements of 20 randomly selected locations with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

All values are expressed as the mean ± SD. Differences in VD, VLD, VDI, and FD values obtained by default and novel segmentation methods were assessed with paired t-tests. A P value less than 0.05 was considered statistically significant.

RESULTS

The mean age of the subjects was 30.7 ± 7.0 years. Segmentations for the RNFL in the central macular area did not show retinal arteries or veins; the most superficial vessels were radial peripapillary capillaries (RPCs; Figs. 1, 2). RPCs were prominent around major retinal vessels, and their VD was significantly higher in the nasal half of the macular area than in the temporal half (Figs. 1, 2). Portions of RPCs extended to the central macular area (Figs. 1, 2). RPCs exhibited relatively straight and long paths, involving few feed points and anastomoses with adjacent retinal vessels (Fig. 2). The mean luminal diameter of the vessels was 22.5 ± 2.7 µm (95% CI: 22.0–23.0).

Segmentations for the GCL visualized the second most superficial vascular layer, which contained capillaries, along with arterial and venous systems. Compared with the slab for SCP in default segmentation, the slab for GCL enhanced visualization of capillary-free zones around the arteries and arterioles, and could easily differentiate arterial and venous systems (Fig. 3). The arteries were subdivided dichotomously, with arterioles branching at right angles from the parent artery (Fig. 3). Precapillary arterioles and postcapillary venules were arranged in an alternating manner and were linked by capillaries, which were arranged in a mesh-like fashion and had multiple arteriolar feed points and anastomoses (Fig. 3). There were no arteriovenous shunts. We defined this compartmentalized collection of arteries (arterioles)-capillaries-veins (venules) as an ACV unit. SCP slabs in the default setting were considered to contain RPCs, GCL vessels, and the top layer of capillaries in the INL. VD in the SCP was significantly greater than in the GCL (Table). Mean luminal diameters of the arteries, arterioles, capillaries, venules, and veins in the GCL were 54.0 ± 12.0 (95% CI: 48.8–59.3), 26.7 ± 3.6 (95% CI: 25.3–28.0), 22.0 ± 3.8 (95% CI: 21.4–22.7), 31.5 ± 3.4 (95% CI: 30.2–32.9), and 53.0 ± 7.1 µm (95% CI: 49.9–56.1), respectively. The slab for the GCL did not show a foveal avascular zone (Fig. 4).

A video comprising sequential-averaged OCT B-scans of the central macular area clearly showed decorrelation signals representing blood flow at the top and bottom of the INL, suggesting the third and fourth vascular plexuses (Supplementary Video S1). Therefore, we performed a novel segmentation to differentiate these two plexuses. Vascular plexuses in the top and bottom of the INL contained units within vortex arrangements (Fig. 4). The VD and FD of the fourth vascular plexuses were significantly higher than those of the third vascular plexus (P = 0.014 and 0.002, respectively, Table). Areas between the units of the vortex arrangements appeared to be watershed areas with low reflectivity (Fig. 4) and corresponded to areas around the arteries and arterioles in the GCL. The center of the vortex arrangements was consistent with that of the venules in the GCL (Fig. 4). OCT B-scan images along the center of the vortex arrangement showed vertical or oblique vessels perpendicular to the retinal plane (Fig. 4).
The foveal avascular zone was delineated in the slab for SCP in the default setting. In contrast, the foveal avascular zone was shown in the slab for the top of the INL in the novel segmentation. Vessel luminal diameters in the top and bottom portions of the INL were 23.1 ± 4.0 (95% CI: 22.4–23.8) and 22.2 ± 3.8 μm (95% CI: 21.5–22.9), respectively; these were equivalent to diameters of RPCs and capillaries in the GCL. VD and FD values for the four vascular plexuses increased with retinal depth (VD, 25.9%, 33.8%, 57.1%, and 58.3%; FD, 1.49, 1.52, 1.59, and 1.60; Table).

**DISCUSSION**

In this study, HR-OCTA allowed isolation of each of the four layers of the retinal vascular plexuses in living human macula and visualization of characteristic features of retinal microcirculation.

The human retina is arranged in layers, and the vasculature in the inner retina is further layered in plexuses that form a retinal neurovascular unit. OCTA enables visualization of these retinal vessel plexuses by segmenting the threedimensional angiographic data into en face slabs. However, available OCTA devices solely provide segmented slabs of the SCP and DCP. Furthermore, most instruments do not tolerate the thinning and termination of inner retinal layers that occurs as the inner retina approaches the center of the fovea. Therefore, the SCP and DCP in the central macula might not be accurately characterized. With electron microscopy, Shimizu and Ujiie examined a cast of monkey retina and showed that the inner retina had three layers of vessel plexuses (RPC, SCP, and DCP). Snodderly et al. subsequently showed that the DCP had two subcomponents, one on either side of...
Henkind et al.2,17 suggested that these capillaries originated from retinal arterioles. Moreover, in a recent study by Fouquet et al.,18 confocal microscopy in a porcine model revealed that RPCs emerged from the SCP and drained to the intermediate capillary plexus or DCP. Confocal observation with adaptive optics could determine how RPCs connect to other retinal microcirculatory units consisting of arterial and venous channels with intervening capillaries. On the basis of our current findings and the results of previous histologic studies10,20,21 we have coined the term “ACV unit” to describe this compartmentalized appearance. To our knowledge, these ACV units have not been demonstrated in living human eyes. In real time, ACV units in the vascular slab for the GCL provide a deeper understanding of retinal circulation than was possible in the past. The ACV unit in the GCL shows that retinal arterioles branch from the parent artery and that (precapillary) arterioles and (postcapillary) venules are arranged in an alternating manner and are linked by capillary beds, consistent with a previous finding involving retinal digest preparation.21 Unlike RPCs, which had a long path length and few arteriolar feed points, capillaries in the GCL were arranged in a mesh-like manner and had multiple arteriolar feed points and anastomoses.

The decorrelation signals on averaged OCTA images for the macrovascular system suggested two-layered vascular plexuses at the top and bottom of the INL (Supplementary Video S1), consistent with previous ultramicroscopic findings in monkey retina.5,15 Therefore, we made an additional segmentation to differentiate the two layers of plexuses in the INL. The INL slab did not include the capillary-free zone around the arteries and arterioles seen in the GCL, but did contain vessels with a vortex arrangement. The center of the vortex arrangements corresponded to the venules in the GCL slab. This finding is consistent with the OCTA findings of Bonnin et al.,22 who reported the presence of polygonal units in the DCP along the course of the superficial venules, but did not demonstrate the presence of the vessels perpendicular to the retinal plane. In our study, an OCT-B scan for the center of the vortex arrangement showed vessels perpendicular to each vessel.
FIGURE 3. Lobular retinal circulatory units highlighted by segmentation of the GCL. Vascular slabs for the SCP in the default setting (A, B) and the GCL (C, D). (B, D) Magnified images of areas enclosed by the squares in (A) and (C), respectively. Compared with the OCTA for the SCP (B), the angiogram for the GCL (D) enhances visualization of the capillary-free zones around the arteries and arterioles, allowing easy differentiation of the arterial (red) and venous (blue) systems. The arteries (red arrows) subdivide dichotomously; the arterioles (red arrowheads) branch at right angles from the parent arteries. The precapillary arterioles and postcapillary venules (blue arrowheads) form an alternating staggered arrangement and are linked by capillaries (asterisks) that are arranged in a mesh-like fashion and have multiple arteriolar feed points (red arrowheads) and anastomoses. There are no arteriovenous shunts. In this study, the compartmentalized appearance composed of arteries/arterioles-capillaries-veins (blue arrows)/venules is referred to as the ACV unit. The colored lines in B-scan images show segmentation lines that were used to generate OCTA (A–D, respectively).

**TABLE.** Differences in Retinal Vessel Parameters between Original and Novel Segmentations in High-Resolution Optical Coherence Tomography Angiography Imaging

<table>
<thead>
<tr>
<th>Segmentation</th>
<th>SCP</th>
<th>RPCN</th>
<th>GCL</th>
<th>DCP</th>
<th>INL Upper</th>
<th>INL Lower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel density, %</td>
<td>38.03 ± 1.46</td>
<td>25.87 ± 7.76</td>
<td>33.82 ± 2.63</td>
<td>38.73 ± 1.15</td>
<td>37.06 ± 1.17</td>
<td>38.33 ± 0.50</td>
</tr>
<tr>
<td>P value</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.003</td>
<td>0.388</td>
</tr>
<tr>
<td>Vessel length density, %</td>
<td>10.04 ± 0.34</td>
<td>7.75 ± 2.60</td>
<td>8.47 ± 0.75</td>
<td>10.51 ± 0.24</td>
<td>10.06 ± 0.32</td>
<td>10.35 ± 0.30</td>
</tr>
<tr>
<td>P value</td>
<td>0.022</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.003</td>
<td>0.183</td>
</tr>
<tr>
<td>Vessel diameter index</td>
<td>3.79 ± 0.11</td>
<td>3.42 ± 0.31</td>
<td>4.00 ± 0.13</td>
<td>3.69 ± 0.08</td>
<td>3.69 ± 0.13</td>
<td>3.71 ± 0.07</td>
</tr>
<tr>
<td>P value</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.978</td>
<td>0.325</td>
</tr>
<tr>
<td>Fractal dimension</td>
<td>1.59 ± 0.01</td>
<td>1.49 ± 0.13</td>
<td>1.52 ± 0.03</td>
<td>1.60 ± 0.01</td>
<td>1.59 ± 0.01</td>
<td>1.60 ± 0.01</td>
</tr>
<tr>
<td>P value</td>
<td>0.044</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.003</td>
<td>0.163</td>
</tr>
</tbody>
</table>

RPCN, radial peripapillary capillary network.
The data are shown as the mean ± standard deviation unless otherwise indicated.
plexus. Mean vessel luminal diameters in the INL were equivalent to the diameters of the capillaries and were less than those of the venules in the GCL. Therefore, we speculate that the vessels with a vortex arrangement in the INL may be prevenule capillaries. With the default setting, the OCT-HS100 delineated the foveal avascular zone in the SCP slab but not in the DCP slab, which occurs when using commonly available OCTA instruments. However, with the novel segmentation method, the foveal avascular zone was shown in the slab for the top of the INL. This difference in delineating the foveal avascular zone reflects a difference in the boundary of each segmentation algorithm. However, visualization of the foveal avascular zone in a slab for the INL in our study supports speculation by Snodderly et al.\textsuperscript{15} that the perifoveal vascular ring is part of the DCP, given that the INL terminates closer to the central fovea than does the GCL.

Our study has some limitations. First, this study included a small study population of only 10 right eyes. Second, each plexus was not perfectly isolated because of the effect of artifacts projected by more superficial retinal vessels. For example, the sum of VD values for the RPC and GCL was greater than that for the SCP. Third, although we could differentiate four layers of vascular plexuses parallel to the retinal plane, we could not visualize connecting vessels perpendicular to the plexuses because of the inherent nature of OCTA en face imaging. Fourth, the imaged 4 × 4-mm\textsuperscript{2} area is not representative of vascularization outside the central macular area. Fifth, segmentation of the four vascular plexuses was not fully automated. Finally, although direct luminal diameter measurements on OCTA imaging were very similar to those measured histologically, measurements for the capillaries were greater; this may have occurred because of the relatively low lateral resolution of OCTA or altered measurements through reflection of the OCT beam.

However, with HR-OCTA, we could differentiate the four layers of the retinal vessel network in living human macula and appreciate the well-regulated and unique features of retinal microcirculation in each plexus. Morphologic differences between RPCs and the mesh-like structures of the other capillaries might cause deflection of blood between RPCs and the deeper capillaries in eyes with increased IOP. Morphologic and functional imbalances in retinal blood flow might occur in the ACV unit in the presence of retinal nonperfusion associated with diabetic retinopathy or retinal vein occlusion. Other research groups have speculated\textsuperscript{23,24} that the deterioration of circulation in the two-layered capillaries in the INL would affect underlying photoreceptors in patients with diabetic retinopathy. Although OCTA cannot completely determine the direction of blood flow, detailed en face observation of vessel morphology with this technique enables detection of both planar and three-dimensional retinal circulation with high accuracy. Development of automated-segmentation software...
that enables differentiation of the retinal arteries and veins and supplementary use of confocal observation with adaptive optics should elucidate both normal physiology and pathologic changes in retinal microcirculation.

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