The retina is one of the most metabolically active tissues in the human body, requiring a complex vascular network to support its high energy demand. Therefore, the retina is especially vulnerable to pathologic vascular changes. Alterations to capillary structure and perfusion have long been observed in retinal conditions such as diabetic retinopathy and are essential for diagnosing and monitoring disease. Consequently, there is high demand for reliable in vivo visualization of the retinal microvasculature that is clinically accessible.

Intravenous fluorescein angiography (IV FA) remains the clinical gold standard for detecting vascular pathology in the retina since Novotny and Alvis first demonstrated this technique in humans in 1961. However, it is an invasive test that requires exposure to an exogenous contrast agent. Though adverse effects of fluorescein are uncommon, they range from nausea and pruritus to anaphylaxis. Moreover, the axial and lateral resolution of conventional IV FA, using traditional fundus cameras and scanning laser ophthalmoscopes, allow for limited visualization of capillaries, especially in areas of the retina where there are overlapping capillary beds.

With the ability to correct for the eye’s monochromatic aberrations, adaptive optics (AO) offers improved resolution of retinal microvasculature over existing clinical imaging modalities. Adaptive optics scanning light ophthalmoscope fluorescein angiography (AOSLO FA) was first validated against histology in the primate retina and has since been successfully demonstrated in human eyes, allowing for better resolution of the vasculature in comparison to conventional IV FA. Recent AO studies have shown that oral fluorescein can be used in place of IV contrast with comparable results, and, because of slower gastrointes-
tional absorption, it has the added advantage of accommodating the longer imaging times currently needed for AO. Though the side effects of oral fluorescein are milder,11,12 anaphylaxis is still a possible consequence of administration.13

Optical coherence tomography angiography (OCTA) has recently emerged as a promising noninvasive way to visualize retinal microvasculature. Instead of exogenous contrast requiring blue light excitation, OCTA uses the motion of erythrocytes illuminated with near-infrared light to generate perfusion maps. The longer wavelength avoids the potential for photochemical damage, which could be especially important for diseased retinas.14-16 Raw OCT data can be processed into OCTA images by using several different techniques including phase-based (e.g., phase variance OCT)16,17 and amplitude-based (e.g., split-spectrum amplitude decorrelation angiography [SSADA])18-21 methods. In comparing OCTA images from human subjects and confocal microscopic images from donor eyes, OCTA reveals the different retinal vascular layers as described in histology and with similar quantitative parameters such as FAZ area.22,23 However, few studies have compared OCTA with other in vivo vascular imaging,24-27 an important step in the validation of OCTA as a clinical tool to be used alongside familiar imaging modalities. Though AOSLO FA is not yet a routine clinical instrument, its ability to produce higher resolution images, compared to conventional IV FA, makes it an ideal modality to validate OCTA. In this study, we sought to evaluate OCTA in imaging the foveal microvasculature in comparison to AOSLO FA.

**METHODS**

**Subjects**

This study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board at the New York Eye and Ear Infirmary of Mount Sinai. Four healthy controls and 11 patients with diabetic retinopathy (four subjects), retinal vein occlusion (four subjects), or sickle cell retinopathy (three subjects) were recruited. Inclusion criteria were as follows: clear natural lens, normal anterior segment, clear media, best corrected visual acuity better than 20/80, good fixation, and pupil dilation to at least 5 mm. Patients with severe macular edema were excluded owing to current limitations of AOSLO imaging and potentially excessive fluorescein leakage obscuring the microvasculature. Healthy controls were defined as having no prior retinal pathologic or major systemic vascular disease. Patient diagnoses were determined from chart review and patient-reported history. Healthy controls were imaged with AOSLO FA and OCTA within 2 years (one subject was imaged on the same day; mean duration between imaging for the other three subjects was 13 months). All vasculopathic patients were imaged with AOSLO FA and OCTA on the same day. Written informed consent was obtained after discussion of the study methodology and risks and benefits. Best corrected visual acuity was assessed before any imaging or pupillary dilation. Pupils were dilated with one drop of 2.5% phenylephrine hydrochloride ophthalmic solution (Paragon BioTeck, Inc., Portland, OR, USA) and two drops of 1% tropicamide ophthalmic solution (Akorn, Inc., Lake Forest, IL, USA). Axial lengths were obtained with an IOL Master (Carl Zeiss Meditec, Inc., Dublin, CA, USA) to account for individual retinal magnification.

**Adaptive Optics Scanning Light Ophthalmoscope Imaging and Image Processing**

The AOSLO used in this study was a replica of the one described by Dubra and Sulai,28 with modification of the visible channel to capture fluorescein signal. Briefly, three light sources with wavelengths of 488, 790, and 850 nm were used for fluorescein excitation, reflectance imaging, and wavefront sensing, respectively.29 A 15-kHz horizontal resonant optical scanner and a 16-Hz vertical optical scanner were used to form a 1.75° square imaging raster. Light exposure was kept below maximum permissible exposure according to the American National Standards Institute ANSI Z136.9-27.

For AOSLO FA, four vials of 10% fluorescein sodium (Akorn, Inc.), or approximately 20 mg/kg, were administered orally with orange juice or water depending on subject preference. Fifteen minutes after fluorescein administration, confocal reflectance and fluorescence sequences with optimal focus of the vascular layer on the fluorescein channel were acquired simultaneously with all three light sources. Subjects’ gaze was directed with a green fixation target, which was moved as needed to image a 6 × 6 (~1.8 × 1.8 mm) area centered at the fovea as previously described.30 Adaptive optics scanning light ophthalmoscope FA image sequences, each with 125 frames, were collected at each retinal location. Additional confocal and nonconfocal (split detection) sequences of the same retinal regions were collected with optimal focus of the vascular layer on the split detection channel.28 Subjects were encouraged to blink throughout the imaging session. Breaks were given approximately every 2 minutes or as needed. Total fluorescein imaging time was approximately 5 minutes, with an additional 5 minutes for confocal reflectance and split detection imaging.

To produce fluorescein images, fluorescence sequences were coregistered with corresponding confocal reflectance sequences as the primary sequence, and 5 to 100 frames with high signal-to-noise ratio from each of the original videos were averaged by using custom software.29 These images were then manually montaged with Adobe Photoshop CS6 (Adobe Systems, Inc., San Jose, CA, USA). Nonconfocal split detection sequences were processed in the same manner.

**Optical Coherence Tomography Angiography Imaging and Image Processing**

Optical coherence tomography angiography imaging was performed by using the AngioVue system on the Optovue RTVue XR Avanti (Optovue, Inc., Fremont, CA, USA). This instrument is a spectral-domain OCT with a scan rate of 70,000 A-scans/s and a scan beam wavelength of 840 ± 10 nm (45-nm bandwidth). Macular 10 × 10 (~3 × 3 mm) scans centered at the fovea were obtained for each subject. Each B-scan was composed of 304 A-scans. Two consecutive B-scans were obtained at each raster location for a total of 608 B-scans per volumetric raster scan, which amounted to an imaging time of 3 to 4 seconds per raster scan. Two raster scans (X-Fast and Y-Fast) were obtained. Perfusion maps were generated from the raw data by using the SSADA algorithm.24,30,31 With custom software, the en face superficial and deep perfusion maps generated by the AngioVue software were superimposed to create a single full microvasculature layer. Since AOSLO FA images the full microvascular layer, this superimposed OCTA image was used to compare the same vascular structures.

**Quantitative Analysis of Microvasculature**

**Skeletonization, FAZ Metrics, and Vessel Density.** To ensure that the regions quantified on corresponding images were the same, OCTA full layer images were registered with AOSLO FA images by using bUnwarpJ (Fig. 1).32 The coregistered AOSLO FA and OCTA images were each semiautomatically skeletonized by using custom software on MATLAB (The MathWorks, Inc., Natick, MA, USA). Briefly, the...
software labels vessels as having a certain threshold pixel intensity and manual cleanup was done by an expert reader (SM). The skeleton data were used to calculate FAZ area, perimeter, and acircularity index (defined as the ratio of the perimeter of the FAZ and the perimeter of a circle with equal area), as well as vessel density for each of the regions of interest (ROIs). Vessel density was defined as the total vessel length divided by the ROI area. The ROIs were defined as concentric rings with inner border at the FAZ margin and outer border at 100, 200, and 300 μm away from the FAZ margin (Fig. 2). Intra- and interexaminer repeatability (second reader: BK) were performed on the FAZ metrics and vessel density at the 100-μm ROI in one randomly chosen set of images from each group.

**Lumen Diameter.** Lumen diameter was measured in 16 to 20 vessel segments in the AOSLO FA and OCTA images before registration with bUnwarpJ. These vessel segments of varying calibers and eccentricities were chosen to cover the broad spectrum of vascular structures seen on the scans (Fig. 3). Using custom software, a reader traces the center of the vessel of interest, ensuring that the sampled segment is at least 30 pixels long on OCTA (corresponding to approximately 40 μm) and 50 pixels long on AOSLO FA (corresponding to approximately 35 μm) (Hillard J, et al. IOVS 2013:54:ARVO E-Abstract 6061). The program straightens the segment and then samples the intensity of the pixels in a predefined ROI around the segment. An average cross-section of the intensities is generated, and the margins of the lumen are defined as the peak and trough in the derivative of the intensity profile. The minimum lengths of the segments were chosen in order to acquire enough pixel intensity data for a representative average curve.

**Qualitative Comparisons of Microvasculature**

Pathologic features noted during imaging were used to highlight the differences between AOSLO FA and OCTA. These included microaneurysms, vessel loops, leakage, and whether or not the vessel segment was identified with the skeletonization program. Differences in vessel identification were also mapped by using the 200-μm ROI skeletonizations. Adaptive optics scanning light ophthalmoscope FA and OCTA images were also compared to AOSLO confocal reflectance and split detection images to identify structural, as opposed to perfusion, differences among these imaging modalities.

**Statistics**

Statistical analysis was performed with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), SPSS 22.0 Statistical Software (IBM Corporation, Chicago, IL, USA), and R version 3.2.3. For all statistical testing, $P < 0.05$ was considered significant. Before hypothesis testing, Anderson-Darling tests for normality were performed on each data set. Sets of data in which normality could not be rejected when using the Anderson-Darling test were analyzed with paired $t$-tests. Sets of data found to be not normally distributed were analyzed with Wilcoxon signed rank tests. Bland-Altman plots were created for each parameter. Intra- and interexaminer repeatability were assessed by using intraclass correlation coefficient (ICC). For the lumen diameter data, a linear mixed model was also generated to adjust for within-subject correlation among measurements and variation in number of measurements collected from each set of images.

**RESULTS**

**Subject Characteristics**

The mean ± SD age of the 15 subjects imaged was 40.7 ± 15.1 years. Six subjects were female (40%). See the Table for additional descriptors.

**Foveal Avascular Zone Metric Agreement Between AOSLO FA and OCTA**

Figure 4 (top row) shows the Bland-Altman plots of the FAZ area, perimeter, and acircularity index. The mean ± SD FAZ
The mean ± SD percentage difference was 1.8% ± 1.2%, which was statistically significant (P = 0.004). The mean ± SD FAZ perimeter was 3.6 ± 1.9 mm on AOSLO FA compared to 3.5 ± 1.6 mm on OCTA. The mean ± SD percentage difference was 3.8% ± 4.1%, which was not statistically significant. The mean ± SD FAZ acircularity index was 1.67 ± 0.39 on AOSLO FA compared to 1.64 ± 0.30 on OCTA. The mean ± SD percentage difference from AOSLO FA to OCTA was 3.7% ± 4.1%, which was not statistically significant. All FAZ metrics showed high intra- and interexaminer repeatability (ICC > 0.9).

**Figure 2.** Adaptive optics scanning light ophthalmoscope FA (top row) and OCTA (bottom row) images of a healthy control subject (RR_0232). (A1, B1) Perfusion maps covering the 300-μm ROI. (A2, B2) Skeletonizations of perfusion maps with AOSLO FA in red, OCTA in blue; missing blood vessel segments seen on the other modality were indicated in yellow. (A3, B3) Density color contour maps produced from the skeletonizations.

**Figure 3.** A selection of corresponding vessel segments with varying lumen diameters on AOSLO FA (top row) and OCTA (bottom row) images in a healthy control subject (RR_0424). (A1, B1) Capillary segment at the FAZ margin. (A2-A5, B2-B5) Arteriolar and venular segments located within 3° from the fovea. Arterial and venules are marked in A and V, respectively.
Table. Subject Characteristics

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* Nonproliferative diabetic retinopathy.
† Proliferative diabetic retinopathy.

Vessel Density Agreement Between AOSLO FA and OCTA

Figure 4 (bottom row) shows the Bland-Altman plots of the vessel densities at each ROI. The mean ± SD densities on AOSLO FA at the 100-, 200-, and 300-μm ROI were $31.5 ± 4.2 \text{ mm}^{-1}$, $32.3 ± 6.0 \text{ mm}^{-1}$, and $34.4 ± 7.1 \text{ mm}^{-1}$, respectively, compared to $31.4 ± 4.3 \text{ mm}^{-1}$, $31.7 ± 6.0 \text{ mm}^{-1}$, and $33.6 ± 6.8 \text{ mm}^{-1}$, respectively, on OCTA. The mean ± SD percentage differences at each ROI were $4.2% ± 3.8%$, $5.2% ± 5.6%$, and $5.6% ± 4.8%$, respectively. None of these differences were statistically significant. Vessel density at the 100-μm ROI showed high intra- and interexaminer repeatability (ICC > 0.9).

Larger Lumen Diameters on OCTA Than on AOSLO FA

A total of 273 vessel segments were measured for each modality (average of 17 per subject). The mean length of vessel segments measured was 102.2 μm on AOSLO FA and 102.6 μm on OCTA. The mean ± SD of lumen diameters measured on AOSLO FA was $14.2 ± 6.3 \text{ μm}$ ($3.6–38.7 \text{ μm}$) compared to $19.9 ± 5.9 \text{ μm}$ ($11–42 \text{ μm}$) on OCTA. The mean ± SD difference between OCTA and AOSLO FA measurements was $5.7 ± 3.2 \text{ μm}$. The Bland-Altman plot (Fig. 5) shows that OCTA measurements were generally larger and that there was no association between the mean lumen diameters and the raw differences. The linear mixed model yielded a highly significant coefficient of $5.7$ for difference in modality ($P < 0.001$), supporting the mean difference from the Bland-Altman plot.
Qualitative Comparisons of AOSLO FA and OCTA

Figure 6 shows representative images of the pathologic features noted on AOSLO FA versus OCTA. Microaneurysms were easily visible on AOSLO FA, but seen variably on OCTA. As shown in Figure 6B, vessels in the area of leakage were obscured on AOSLO FA, but no leakage was detected on OCTA as observed in previous studies and therefore these vessels were visible. Vessel loops were present in both modalities but some of the finer structure was better captured on AOSLO FA. Some capillary segments were visualized on AOSLO FA but not on OCTA, likely owing to slow flow below the detection velocity threshold of 0.03 mm/s (Fig. 6D).

As shown in Figure 7, significant differences in vessel identification were observed between the two modalities. Notably, most of the vessel segments missing on AOSLO FA compared to OCTA were shorter than those missing on OCTA compared to AOSLO FA. Additionally, some of the latter differences were in vessels that appear to run very close to ones that were already identified on OCTA.

Figure 8 highlights the ability of AOSLO confocal reflectance and split detection imaging to capture nonperfused vessels that were neither visible on AOSLO FA nor OCTA in a subject with proliferative diabetic retinopathy (RR_0449). On the confocal image, these nonperfused vessels appeared less evenly reflective, while on the split detection image, they appeared flatter and had thinner walls.

DISCUSSION

In this study, we examined the similarities and differences between AOSLO FA and OCTA, finding good agreement in FAZ metrics and vessel density. Lumen diameter was, however, generally larger on OCTA than on AOSLO FA, likely owing to lower sampling and image resolution (diffraction) and/or low-pass filtering within or after the SSADA algorithm in the former. Qualitative differences included visibility of microaneurysms, fine structure of vessel loops, leakage, and some capillary segments. These differences may be due to limitations in OCTA such as its detection velocity threshold and resolution.
FIGURE 7. Skeletonizations of AOSLO FA (top row, red) and OCTA (bottom row, blue) images covering the 200-μm ROI in (1) a healthy control subject (RR_0424) and (2) a diabetic retinopathy subject (RR_0217). Missing vessel segments compared to the other modality are highlighted in yellow.

FIGURE 8. Imaging nonperfused capillaries in a subject with diabetic retinopathy (RR_0449) using AOSLO. Nonperfused capillaries (yellow arrows) are clearly visible on AOSLO confocal reflectance and AOSLO split detection (bottom row) but not on OCTA and AOSLO FA (top row).
The major limitations of this study were as follows: (1) the sample was small and nonrandom secondary to strict AOSLO selection criteria, particularly regarding patients with severe macular edema or medial opacities; (2) the AOSLO image processing and analysis using skeletonization are tedious and not necessarily accessible; and (3) the area imaged was limited to the perifovea.

Capillary dropout is a prominent feature of vasculopathic retinal diseases. Changes in the FAZ have been shown to be a good indicator of capillary dropout and are associated with disease progression. In particular, enlargement of the FAZ seems to be correlated with reduced visual acuity.\(^{41,42}\)

Therefore, the ability to track changes in FAZ structure is important for monitoring the progression and treatment of these diseases. The area, perimeter, and acularity reported here are consistent with previous studies on healthy and diseased eyes.\(^{29,34,35,41,43–52}\)

The small but statistically significant difference of 1.8% in area could be explained by differences in detection of capillary segments that make up the FAZ margin, especially in vasculopathic eyes. This finding is likely due to blood flow that is slower than the detection velocity threshold of 0.3 mm/s on OCTA\(^ {53}\) or intermittent flow through these capillaries. Diabetic and sickle cell patients have been shown to have slower blood flow velocity in perifoveal capillaries as compared to healthy individuals.\(^ {42,44,50,54–56}\)

Similar observations have been made in the vascular distributions affected by retinal vein occlusion.\(^ {57}\)

We observed that there are a number of vessel segments missing on OCTA compared to AOSLO FA. There are several sources for this difference. First, AOSLO FA has higher spatial sampling and lateral resolution than OCTA. Fine structures such as two closely adjacent capillaries or vessel loops seen on AOSLO FA may appear on OCTA to be a single vessel or a microaneurysm, respectively. Thus, OCTA is not an ideal tool to monitor changes in these fine structures. Second, similar to the reasoning behind the difference in FAZ area, some of the capillaries may have intermittent flow or slower blood flow velocity, especially in vasculopathic eyes. This observation raises an interesting clinical application; in combination with exogenous contrast-enhanced imaging such as IV FA, OCTA has the potential to identify vessels at risk for complete occlusion, since they will appear faint or absent on OCTA but apparent with exogenous contrast.

Compared to AOSLO FA, OCTA is more prone to eye motion artifacts and has lower lateral resolution, resulting in the differences in lumen diameter and appearance of vessels between the two modalities. Since the sources of the perfusion signal (i.e., fluorescein for AOSLO FA and red blood cells for OCTA) are confined to the intraluminal space, the images produced should represent the vessel lumen. Lumen diameters of retinal capillaries near the FAZ in histologic and previous AO studies are approximately 5 to 10 \(\mu m\),\(^ {60,61}\) which is in agreement with the present AOSLO FA data. Yet, as this and previous studies have shown, vessels on OCTA look more heterogeneous, do not have a smooth appearance, and have larger diameter than on histology or AOSLO.\(^ {22,62–64}\)

In agreement with previous studies,\(^ {59,60}\) we found differences in the visualization of leakage and microaneurysms on OCTA compared to AOSLO FA. Leakage has been shown to be important in the diagnosis, monitoring, and treatment of a number of retinal diseases, including diabetic retinopathy and retinal vascular occlusions; microaneurysm formation also contributes to the diagnosis of retinal vasculopathy, and detection of leaking microaneurysms on IV FA can direct localized treatment such as laser photocoagulation for macular edema.\(^ {65–73}\)

Since OCTA is unable to consistently show these pathologic features, owing to slow or intermittent blood flow, it may be useful to pair it with another modality such as fundus photography or IV FA for more complete clinical assessment. Vascular changes in which there is adequate flow velocity are easily visible with OCTA and can be localized axially.\(^ {59}\)

For example, Figure 10 shows that the dilated vessels in two branch retinal vein occlusion patients are located primarily in the deep plexus. This ability to axially locate pathologic vascular changes may help further our understanding of the natural history of retinal diseases.
CONCLUSIONS
Optical coherence tomography angiography foveal microvascular images appear comparable to AOSLO FA. With its ease of use, noninvasive nature, and short scan time, OCTA is an attractive option for retinal disease screening. In addition, OCTA uniquely delineates multiple capillary layers in a single volumetric scan and is able to reveal blood vessels obscured by leakage. In its current form, however, low scanning speed and resolution limit its ability to visualize important pathologic features such as microaneurysms, leakage, and vessel loops. Technologic improvements, such as eye motion compensation, could lead to its broad adoption as a noninvasive method for detecting retinal vascular disease.

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