

Correlation of Histologic and Clinical Images to Determine the Diagnostic Value of Fluorescein Angiography for Studying Retinal Capillary Detail

Kanishka R. Mendis,¹ Chandrakumar Balaratnasingam,^{1,2} Paula Yu,^{1,2} Chris J. Barry,¹ Ian L. McAllister,¹ Stephen J. Cringle,^{1,2} and Dao-Yi Yu^{1,2}

PURPOSE. To delineate morphometric and quantitative features of the capillary image derived from high-resolution fundus fluorescein angiography (FFA) and consequently determine the diagnostic value of FFA for studying the retinal capillary circulation.

METHODS. Retinal capillary images obtained from healthy young subjects using high-resolution FFA were compared with confocal scanning laser microscopic capillary images derived from the retinas of age-matched human donors. Confocal microscopic images were acquired from retinal flatmount tissue after central retinal artery cannulation, perfusion fixation, and antibody labeling. Capillary images from equivalent retinal regions were morphologically and quantitatively analyzed in both groups.

RESULTS. Ten human subjects (mean age, 27.4 years) were used for FFA studies, and five cadaveric eyes (mean donor age, 26.5 years) were used for histologic studies. In histologic specimens the density of the superficial capillary network was significantly greater than that of the deep capillary network. Despite use of a healthy young population, only 30% of high-resolution FFA studies provided clear capillary images. The configuration of the capillary network in FFA images was comparable to the superficial capillary network in confocal microscope images; however, the density of the capillary network in FFA images was consistently lower than that of histologic images.

CONCLUSIONS. FFA provides incomplete morphologic information about the superficial capillary network and even less information about the deep capillary network. Caution should, therefore, be exercised when using FFA data to extrapolate information about microvascular histopathologic processes. The usefulness of newer technology for studying retinal capillary detail should be investigated. (*Invest Ophthalmol Vis Sci.* 2010;51:5864–5869) DOI:10.1167/iops.10-5333

From the ¹Centre for Ophthalmology and Visual Science and the ²ARC Centre of Excellence in Vision Science, University of Western Australia, Perth, Australia.

Supported by the National Health and Medical Research Council of Australia and the Australian Research Council Centre of Excellence in Vision Science.

Submitted for publication February 6, 2010; revised April 2, 2010; accepted May 14, 2010.

Disclosure: **K.R. Mendis**, None; **C. Balaratnasingam**, None; **P. Yu**, None; **C.J. Barry**, None; **I.L. McAllister**, None; **S.J. Cringle**, None; **D.-Y. Yu**, None

Corresponding author: Dao-Yi Yu, Centre for Ophthalmology and Visual Science and the ARC Centre of Excellence in Vision Science, The University of Western Australia, Perth, Australia; dyyu@cyllene.uwa.edu.au.

Since its introduction into clinical practice more than 40 years ago,¹ the technique of fundus fluorescein angiography (FFA) has gained broad application for the diagnosis and management of ophthalmic disorders. FFA is known to allow accurate delineation of large- and medium-caliber retinal vessels,¹ but its ability to allow reproducible delineation of retinal architecture at the capillary level remains unclear. Despite this lack of knowledge, FFA data are frequently used to stratify and manage microvascular retinal diseases^{2,3} that are collectively a major cause of visual morbidity worldwide.⁴ Previously, the lack of high-resolution cameras and sophisticated imaging software might have posed serious limitations on the level of capillary detail that could be derived from FFA, but with the availability of improved state-of-the-art technology, many of these limitations can now be addressed.

A large portion of the human retina is nourished by an elaborate, multilayered network of capillaries that can be broadly classified into two groups, superficial and deep. These two networks are morphologically distinct and individually adapted to suit the functional demands of their retinal environment.⁵ In the peripheral retina, histologic studies have demonstrated a superficial network of capillaries located in the plane of the nerve fiber layer and a deep network of vessels situated in the boundary plane between the inner nuclear layer and the outer plexiform layer.^{5,6} At the posterior pole, however, the capillary pattern within the thicker retina is considerably different. The deep capillary network is almost unaltered in morphology compared with the peripheral retina, but the superficial network demonstrates a complex three-dimensional configuration because of the interconnection of multiple capillary loops at the superior boundary of the inner nuclear layer.⁷ Selective capillary network dysfunction may be an important determinant of clinical disease progression and may also be inherently linked to critical pathogenic mechanisms that modulate retinal vascular diseases.⁸ The amount of superficial capillary network and deep capillary network detail that can be reliably derived from human retinal angiograms, however, remains unknown. Understanding the level of capillary detail that can be extracted from FFA is important because it may assist the clinician to more accurately infer knowledge about retinal histopathologic processes using FFA data. It may also identify limitations in the application of current FFA techniques for diagnostic and investigative purposes.

This study explored the usefulness of high-resolution FFA images for the examination of human retinal capillary networks and the factors that influence accurate discernment of capillary detail in fluorescein angiograms. We used our previously described micropipette technique⁹ to selectively label the retinal circulation and subsequently used confocal microscopy to study the different capillary networks within the retina. Morphologic and quantitative comparisons were then made between histologic and age-matched FFA data of healthy young

eyes to determine whether the superficial capillary network, deep capillary network, or both are identifiable on high-resolution fluorescein angiograms. We used quantitative measurements collected from these experiments to make comparisons between the human retinal microcirculation and the monkey retinal circulation described previously.^{6,10,11} The purpose of this study was to define the boundaries within which information about retinal capillaries can be reliably extrapolated from FFA images. The results may be used to improve FFA interpretation in clinical practice.

METHODS

This research followed the tenets of the Declaration of Helsinki and was approved by the University of Western Australia human ethics committee. Correlation of retinal angio-architecture was performed using data collected from male subjects who underwent retinal fluorescein angiography and age-matched cadaveric eyes that underwent histologic examination with confocal scanning laser microscopy.

Retinal Fluorescein Angiography

Ten healthy male volunteers (mean age, 27.4 ± 1.8 years; range, 21–39 years) were subject to fluorescein angiography. Informed consent explaining the nature and possible consequences of the study was obtained from all participants before recruitment into the study. Only one eye from each subject was used. Enrolled subjects had no history of ocular or medical disease. Subjects with a positive family history of any ocular disease were also excluded from this study. Details of subjects used in this study are provided in Table 1.

After pupil dilation with tropicamide 1% and phenylephrine hydrochloride 10%, baseline color fundus photographs and autorefractometry were performed on all subjects. Fluorescein angiography was performed using a bolus injection of 5 mL of 10% sodium fluorescein (Phebra, Sydney, Australia) administered through the antecubital vein. Angiogram images were captured using a digital fundus camera (CF-60DSi; Canon Inc., Tokyo, Japan) at a 40° angle of view, which resulted in a camera magnification factor of 2.5. A camera (EOS 5D; Canon Inc.) with a full-frame complementary metal oxide semiconductor (CMOS) sensor with 12.8 megapixels resolution was used as the capture device using Digital Health Care (Cambridge, UK). All images were taken by a senior ophthalmic photographer (CJB).

Confocal Laser Scanning Microscopy

Five eyes from four donors (mean age, 26.5 ± 2.7 years; range, 19–32 years) were used. All eyes were obtained from the Lions Eye Bank of Western Australia (Lions Eye Institute, Western Australia) after removal

of corneal buttons for transplantation. Donor eyes used for this research had no documented history of eye disease. Demographic data of each donor are presented in Table 1.

Our previously published technique of central retinal artery cannulation, perfusion, and staining was used to examine the retinal circulation microscopically.⁹ In brief, the central retinal artery of each eye was cannulated, perfused with AlexaFluor 546 phalloidin (A22283; Invitrogen, Carlsbad, CA), fixed, and flatmounted. This permitted selective labeling of microvascular mural cells. Retinal tissue was imaged (Plan Apo; Nikon, Tokyo, Japan) with a ×10/0.45 objective lens and a confocal scanning laser microscope (C1; Nikon, Tokyo, Japan). Optical sectioning began at the vitreal surface and terminated at the choroidal surface. Collecting a full-thickness retinal Z-stack allowed us to examine all the different retinal vascular layers. Z-stacks were captured using Kalman averaging, slices within the stack were separated by a distance of 1.1 μm. The resultant optical stack was then separated into a superficial vessel layer and a deep vessel layer before analysis.

Image Analysis

Quantitation of all images was performed using image analysis software (Image Pro Plus, version 5.1; Media Cybernetics, Herndon, VA) and image editing software (Photoshop [version 8.0] and Illustrator CS2 [version 12.0]; Adobe Systems Inc., San Jose, CA). Before analysis, all fluorescein images were graded based on the calculated ratio between vascular and background fluorescence. High-Gaussian and high-pass filters were applied to fluorescein images to improve contrast between vascular and background fluorescence. The following grading scheme, based on the ratio of mean vascular to background fluorescence, was used to grade all images (Fig. 1): grade 1, >4:1; grade 2, <4:1 and >2:1; grade 3, <2:1. Only grade 1 fluorescein images were used for quantitative analysis of retinal angio-architecture and density.

Capillary density and morphologic analysis was performed in equivalent retinal regions in both fluorescein angiogram and histologic specimens. Using the center of the foveal avascular zone (FAZ) as a reference point, images with dimensions 1300 × 1300 μm, with the center point situated 1500 μm from the reference point, were analyzed (Fig. 2). A region temporal and a second region inferior to the reference point were analyzed per eye. Temporal and inferior regions were analyzed because preliminary work in our laboratory demonstrated that capillary image quality in these regions was greater than the quality of images captured from retinal tissue situated nasal and superior to the fovea.

Distances on microscopic images were determined after lens calibration. For fluorescein angiogram images, we applied Littman's formula to convert pixel measures to anatomically correct dimensions.^{12,13} This technique ensured that regions analyzed in fluorescein angiogram and histologic images were reliably matched. Retinal vessels were manually traced, the proportion of the image occupied by retinal vessels was expressed as a percentage, and the unit of measurement was calculated as the percentage retinal area occupied by capillary network.

For patients B and H, we performed capillary density analysis at 16 seconds, 18 seconds, and 22 seconds in the FFA sequence to determine whether angiographic vessel density was modified as a function of time. We also combined the FFA frames for the three time points and measured capillary density. The results of these analyses are provided in Table 2. Similar to what has been reported in monkey retina,¹¹ we did not find a significant difference in capillary density values at different time points after fluorescein injection (*P* = 0.216). Because estimates of angiographic capillary density were not substantially increased by studying multiple FFA frames, we used only data acquired from the 18-second time frame for statistical comparisons against confocal microscope data. Capillary density measurements in combined frames was, on average, 12.5% higher than the 18-second time frame; however, this was not a significant difference (*P* = 0.105).

TABLE 1. Patient and Donor Data

Subject	Sex	Age (y)	Eye	Refractive Error	Image Grade
Normal					
A	M	30	R	Emmetropia	3
B	M	29	L	−2.75DS	1
C	M	21	R	Emmetropia	2
D	M	21	R	−6.0DS/−2.25DC	1
E	M	21	L	−2.75DS/−0.50DC	2
F	M	39	R	Emmetropia	2
G	M	25	R	Emmetropia	3
H	M	28	R	Emmetropia	1
I	M	32	L	−1.0DS	3
J	M	28	R	Emmetropia	1
Donor					
K	M	28	R+L	N/A	N/A
L	F	19	L	N/A	N/A
M	M	32	L	N/A	N/A
N	M	27	L	N/A	N/A

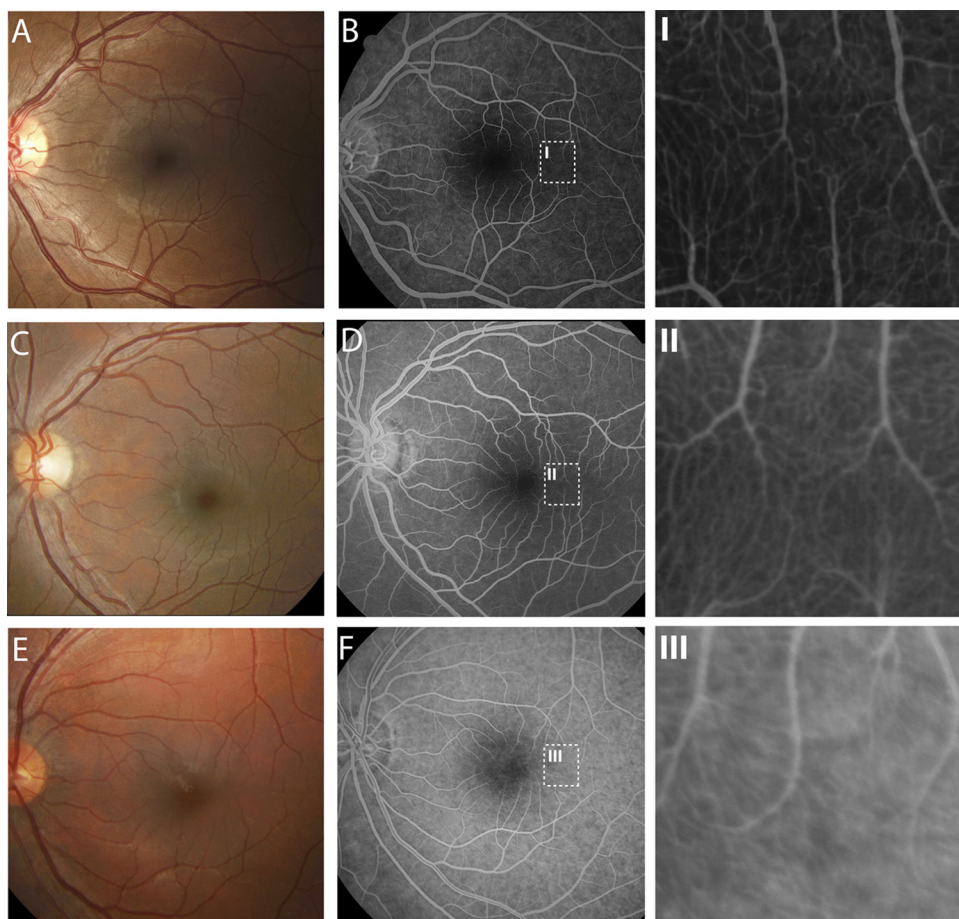


FIGURE 1. Grading of fluorescein angiogram images. Color fundus and fluorescein angiogram images demonstrate the variability in background fluorescence among healthy young subjects. (A, B, *inset I*) High contrast in fluorescence between vascular and background tissue (grade 1, ratio $>4:1$). (C, D, *inset II*) Lower contrast in fluorescence between vascular and background tissue (grade 2, ratio $4:1-2:1$). (E, F, *inset III*) Very low contrast in fluorescence between vascular and background tissue (grade 3, ratio $<2:1$).

Reproducibility of Measurements

To determine observer reproducibility, all fluorescence and confocal microscope images were quantified on two separate occasions, each at least 1 week apart, by the same masked observer who performed all the data analysis.

Statistical Analysis

All statistical testing was performed using commercial software (SigmaStat, version 3.1; SPSS, Chicago, IL). Kolmogorov-Smirnov testing was performed on all data before analysis to determine whether data were normally distributed. Normally distributed data were analyzed using ANOVA with post hoc factor comparison performed using a paired Student's *t*-test with Bonferroni correction. Non-normally distributed data were analyzed using ANOVA on ranks, with the Tukey test used for post hoc paired analysis. Results are expressed as mean \pm SE.

RESULTS

FFA Image Quality

Using a senior ophthalmic photographer to capture posterior segment photographs ensured that the optical quality of all fluorescein angiograms were of a high standard. The inclusion of only healthy young subjects in this study also reduced the influence of media opacities and pupil abnormalities on fluorescein angiogram image quality.

In all 10 eyes, the single-layered capillary region that demarcated the foveal avascular zone could be clearly identified on fluorescein angiograms. However, in the retinal regions that were analyzed in this study, in which multi-layered capillary

networks are known to exist, we were able to make meaningful and reliable analysis of capillary morphology and density in only 30% of images. The presence of choroidal fluorescence significantly restricted our ability to accurately delineate capillary detail in a large number of images. Of the seven images excluded from analysis, three were classified as grade 2 and four were classified as grade 3 (Fig. 1).

Histologic Image Quality

Clear labeling of vascular mural cells, using our novel technique of central retinal artery cannulation and antibody perfusion, was clearly evident on confocal scanning laser microscopy images. There was a high level of contrast between vascular tissue and background tissue in these images. Collecting a Z-stack of images through the entire thickness of the retina also permitted us to accurately divide the capillary networks into a superficial and deep system. We found that in our histologic specimens, the superficial capillary network was situated within the inner 50 μm (mean 30 μm) of retina.

Capillary Morphology and Density Measurements in Histologic Sections

Both the superficial capillary network and the deep capillary network were arranged in a well-defined layered pattern (Figs. 3B, 3C). Individual capillaries in both networks were relatively homogeneous and interconnected by anastomotic capillary bridges in the absence of true laminated structures (Figs. 3B, 3C). There were, however, distinctive differences in vascular pattern and mesh distribution between the two networks. We observed the deep capillary network to be sparser in density

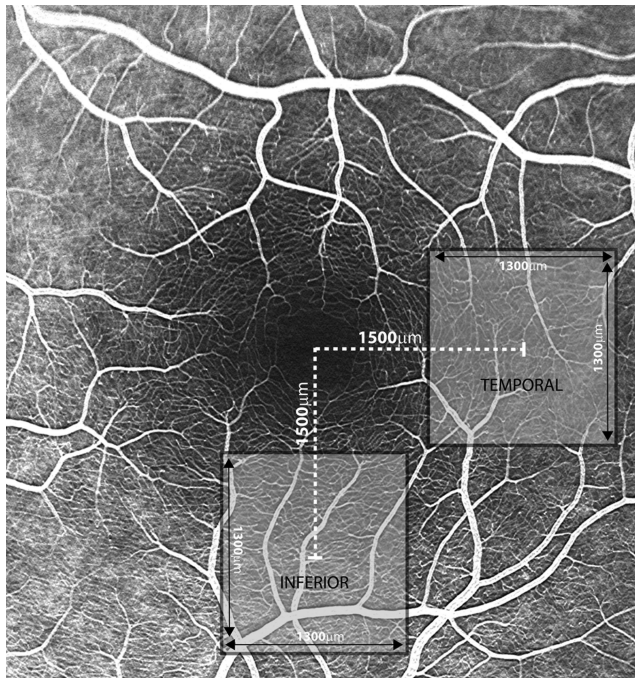


FIGURE 2. Fluorescein angiogram demonstrating the method of retinal image segmentation for capillary density analysis. Regions that were situated temporally and inferiorly in relation to the center of the FAZ were chosen for analysis in both fluorescein angiogram and flat-mount histology images. The region of analysis measured 1300 × 1300 μm in dimension, with the center situated 1500 μm from the center of the FAZ.

than the superficial capillary network. Examination along the z-axis of the confocal stack also highlighted the planar configuration of the deep capillary network that was similar in morphology to a two-dimensional net (Fig. 3C). In contrast, the superficial capillary network was multilayered and less perfectly two-dimensional than the deep network. With regard to mesh pattern, we observed the shape of a single mesh in the deep capillary network to be generally flatter and more elongated than the superficial capillary network such that it produced a rectangular configuration (Fig. 3C).

Mean capillary density in the temporal region of analysis was 41.0% ± 2.5% retinal area occupied by capillary network in the superficial capillary network and 22.4% ± 2.1% retinal area occupied by capillary network in the deep capillary net-

TABLE 2. Density Measurements in Different Frames of FFA Sequence

	FFA Frame Sequence			
	16 Seconds	18 Seconds	22 Seconds	Combined Frames
Patient B				
Inferior	24	23	19	26
Temporal	23	24	20	27
Patient H				
Inferior	22	23	24	25
Temporal	26	26	28	30

Data are expressed as percent retinal area occupied by capillary network. There was no significant difference in capillary density between 16-s, 18-s, and 22-s frames ($P = 0.216$). Combining the three frames yielded a nonsignificant, average increase in capillary density of only 12.5% from the 18-s frame.

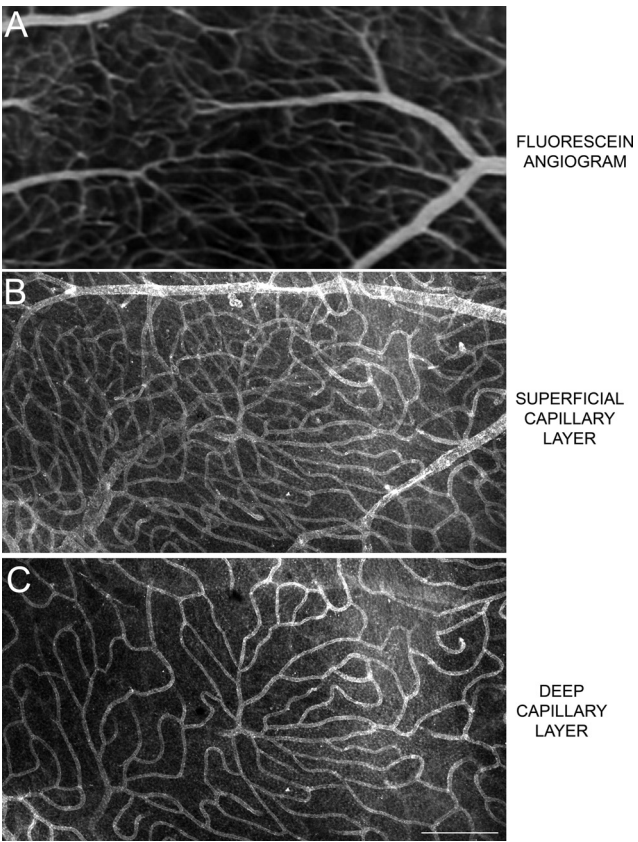


FIGURE 3. Comparison of capillary morphology between fluorescein angiogram image (A), histologic image of the superficial capillary layer (B), and histologic image of the deep capillary layer (C). Scale bar, 200 μm.

work. Mean capillary density in the inferior region of analysis was 41.2% ± 2.2% retinal area occupied by capillary network in the superficial capillary network and 23.5% ± 2.3% retinal area occupied by capillary network in the deep capillary network. There was no difference in capillary density measurements between donor eyes ($P = 0.068$). Capillary density was greater in the superficial capillary layer than in the deep capillary layer ($P < 0.001$). Repeated analysis of all images did not reveal a significant difference between day of measurement and capillary density ($P = 0.928$).

Capillary Morphology and Density Measurements in Fluorescein Angiograms

Morphology of the capillary network seen on fluorescein angiography appeared to be less dense than that seen on histologic specimens (Fig. 3A). In particular, we observed incomplete filling of many capillary meshworks on FFA. The pattern of the capillary meshwork on FFA was also observed to be morphologically similar to the superficial capillary network seen in confocal images. Mean capillary density in the temporal region of analysis was 23.6% ± 1.6% of the retinal area occupied by the capillary network; in the inferior region of analysis, it was 25.1% ± 1.3% of the retinal area occupied by the capillary network. There was no significant difference in capillary density measurements between subjects ($P = 0.108$). Repeated analysis of all images revealed a significant difference between day of measurement and capillary density ($P = 0.027$). The mean difference between the two sets of measurements was 3.9% ± 0.6% of the retinal area occupied by the capillary network.

Comparison of Capillary Density between Fluorescein Angiography and Histology

The capillary density of fluorescein angiogram images was significantly lower than that of the superficial capillary layer on histologic specimens. This was a consistent difference found in both sets of quantitative measurements performed on separate dates (both $P < 0.001$). There was no difference in capillary density measurements between fluorescein angiography and the deep capillary layer of histologic specimens. This difference was not significant in either set of quantitative measurements that were acquired on separate dates (both $P > 0.520$).

DISCUSSION

This study reaffirms the presence of multiple capillary networks within the human retina. Using flatmount, antibody-labeled retinal sections, we clearly demonstrated the dissimilarity in vessel morphology and capillary density between superficial and deep capillary layers. We reiterate, however, that the purpose of this work was not to quantify the number of capillary layers that constituted the three-dimensional configuration of superficial capillary networks. Detailed histologic studies by Snodderly et al.^{6,10} have previously delineated the multilaminar capillary architecture of the nonhuman primate retina. These previous studies confirmed that capillary density varied with retinal eccentricity and was proportional to nerve fiber layer thickness. Interpolation of data from these studies provides a total capillary density estimate of approximately 40% at retinal eccentricity 1500 μm from the fovea.^{6,10} By using confocal scanning laser microscopy techniques to capture images of the retinal circulation along the z-axis, we calculated mean capillary density values of 41.1% for the superficial capillary layer and 23.0% for the deep capillary layer at an equivalent human retinal eccentricity. The higher total capillary density values in our study compared with those of Snodderly et al.^{6,10} is most likely the result of species differences between the two reports. The human retinal nerve fiber layer is thicker than that in nonhuman primates,^{14,15} and our calculated capillary density values may reflect the greater nerve fiber layer thickness in humans. Results of the present study, therefore, support previous findings by Snodderly et al.^{6,10} but also provide separate estimates for the superficial and deep capillary layers in the human retina. The greater density of capillary structures in the superficial layer of the retina may be indicative of critical metabolic and functional differences between the inner and outer layers of the retina in the two species. Because of the technical complexities associated with microvascular cannulation and perfusion—hence, the restricted sample size of this study—we were unable to explore the impact of variables such as age and sex on retinal capillary morphology.

Weinhaus et al.¹¹ have previously performed an in-depth comparison of microvascular information attained from fluorescein angiography and retinal histology using macaque retinas. Their experiments demonstrated that FFA capillary detail declined proportionally with distance away from FAZ. In addition, they clearly showed that capillary visibility on FFA was a joint function of retinal capillary diameter and retinal depth, with large diameter capillaries more clearly visualized on FFA than small diameter capillaries. Weinhaus et al.¹¹ also reported that capillary segments in the nerve fiber layer were visualized more than four times as effectively as segments of comparable diameter in the deepest vascular plane. In their work, >50% of superficial capillaries were visualized out to an eccentricity of 1200 μm , whereas <20% of deep capillaries were visualized in equivalent retinal regions.¹¹ Our experiments demonstrate that FFA capillary density measurements equate to approximately 50% of superficial capillary density values obtained from histologic specimens. Morphologically, vascular patterns seen on

FFA in our study were consistent with the superficial capillary network and not the deep capillary network. Poor visualization of the deep capillary network in FFA in our study was most likely the result of light scatter by the inner retinal layers, resulting in image degradation of deeper retinal layers. This phenomenon has been previously demonstrated by Gorrand et al.¹⁶ We attempted to minimize the deleterious optical effects of nonvascular tissue by examining retinal regions that were situated inferior and temporal to the fovea, where nerve fiber layer thickness and glial content are known to be lower than in superior and nasal regions¹⁷; however, we were still unable to obtain clear images of the deep capillary layer on FFA. Again, the greater thickness of the nerve fiber layer in humans,¹⁴ resulting in greater light scatter, may explain why we obtained less FFA information about the deep capillary layer than did Weinhaus et al.,¹¹ who examined monkey retina.

Clinical studies that can identify capillary structure and density would certainly be useful because it is capillary flow that is key to adequate perfusion. Important morphometric differences between the human retina and the monkey retina, such as nerve fiber layer thickness and photoreceptor density, may affect the level of capillary detail seen on FFA in each species. Consequently, without performing work in humans, it may not be possible to determine how reliably microvascular detail on FFA, acquired from monkey studies, can be extrapolated to human eyes. The results of our study, taken together with what has previously been reported in nonhuman primates, outline the limitations of FFA in clinical practice. The results of this study also demonstrate that despite advancements in FFA technology in the past 15 years, since the publication of the study by Weinhaus et al.,¹¹ the presence of choroidal fluorescence still poses a significant limitation to interpreting capillary detail using FFA images. Another significant contribution this study makes to the ophthalmic literature is the quantitative and qualitative information it provides about the superficial and deep capillary layers in the healthy human retina. The techniques presented also offer the potential to evaluate new instruments, such as the fluorescence adaptive optics scanning laser ophthalmoscope, which has recently offered improved resolution in monkey studies.¹⁸ There is a paucity of knowledge concerning the differences in structure among the various retinal capillary layers. We have used our microvascular cannulation techniques to improve our understanding of these differences.

The present study used complex microvascular perfusion techniques, together with confocal scanning laser microscopy, to delineate qualitative and quantitative information about the different capillary layers in the retina. To our knowledge this has not been previously reported in human eyes. Taken together with previous findings by Weinhaus et al.¹¹ and Snodderly et al.,^{6,10} we have been able to identify significant similarities and differences between human and monkey retinal capillary networks that may aid our understanding of functional distinctions between the two species. Importantly, the present study demonstrates that choroidal fluorescence poses a significant negative influence on the observer's ability to delineate the retinal capillary circulation despite the use of modern and high-resolution FFA equipment. We analyzed FFA images from healthy young subjects and were able to make meaningful interpretation of capillary detail in only 30% of participants. The major limitation in FFA image analysis identified in this study is the poor ratio between capillary signal and background noise, not image resolution. In clinical practice in which FFA is commonly performed in an older group of patients, the ability to analyze capillary detail based on FFA data would be expected to be even lower because of the presence of optical media opacities and pupil disorders, which may further degrade the quality of the angiogram.

The results of this study suggest that FFA provides incomplete morphologic information about the superficial capillary layer and even less information about the deep capillary layer. They also suggest that the usefulness of FFA for studying capillary detail is no better than it was 15 years ago,¹¹ despite the increased usefulness of newer FFA technology for studying medium and large vessel chorioretinal diseases.¹⁹ It is possible that the lower capillary density values attained from FFA, compared with histology, reflect the unstable nature of the retinal microcirculation. Work in our laboratory has demonstrated that the retinal circulation has a considerable capacity to regulate blood flow as tissue demand or arterial input changes.^{20–22} We did not find a significant difference in capillary density values when FFA frames at different time points were measured; however, it is possible that changes in capillary density may become apparent if FFA frames separated by larger time intervals were analyzed. It is not feasible to perform consecutive angiograms on the same human subjects for research purposes, although this issue may be explored in animal studies. The complexity of the capillary architecture, together with the lower resolution in FFA images, resulted in a mean difference of 3.9% between repeated sets of measurements. Although this difference was statistically significant, on both occasions, the density measurements in FFA data were significantly lower than in histology data. Previous work examining capillary morphology using imaging techniques have also demonstrated some variability after repeated measures of complex vascular morphology.²³

Retinal microvascular disease, particularly diabetic retinopathy, remains a major cause of visual morbidity and blindness worldwide.²⁴ Clinical detection of early microangiopathy in this group of conditions remains a challenge. Recent work has focused on the development of digital image processing algorithms as a means of identifying early retinal disease at the capillary level.^{25–27} If successful, this would permit the clinician to better stratify disease severity and administer timely therapeutic intervention before the onset of irreversible, sight-threatening complications. Although there is little doubt that conventional FFA has an important role in diagnostic ophthalmology, the present study demonstrates that FFA is limited in its capacity to provide information about retinal capillaries. Technology that permits confocal scanning of the retina has been developed, but few studies have been conducted to investigate the ability of these devices to reliably outline capillary structures.²⁸ One of the potential benefits of these newer devices may be their ability to eliminate the deleterious effects of choroidal fluorescence and, hence, allow selective imaging of retinal vessels. Further work will be required to explore the usefulness of newer retinal imaging devices for studying retinal capillary detail in the human eye.

Acknowledgments

The authors thank the staff of the Lions Eye Bank of Western Australia, Lions Eye Institute, for providing human donor eyes and the staff of DonateWest, the Western Australian agency for organ and tissue donation, who facilitated the recruitment of donors into the study by referral and the completion of the consent processes.

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