Title: Quantitative morphometry of perifoveal capillary networks in the human retina

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Abstract

Purpose: To quantify the distribution and morphometric characteristics of capillary networks in the human perifovea. To determine correlations between the location of neuronal subcellular compartments and the morphometric features of regional capillary networks in the layered retina.

Methods: The perifoveal region, located 2 mm nasal to the fovea, was studied in 17 human donor eyes. Novel micropipette technology was used to cannulate the central retinal artery and label the retinal microcirculation using a phalloidin perfusate. Gamma-synuclein, goalpha and parvalbumin antibodies were also used to co-localize the nerve fibre layer (NFL), retinal ganglion cell layer (RGCL), inner plexiform layer (IPL) and inner nuclear layer (INL). Confocal scanning laser microscopy was used for capillary imaging. Capillary diameter, capillary density and capillary loop area measurements were compared between networks.

Results: Four capillary networks were identified in the following retinal layers: (1) NFL (2) RGCL and superficial portion of IPL (3) Deep portion of IPL and superficial portion of INL (4) Deep portion of INL. Laminar configurations were present in NFL and deep INL networks. Remaining networks demonstrated three-dimensional configurations. Capillary density was greatest in the networks serving the IPL. Capillary loop area was smallest in the two innermost networks. There was no difference in capillary diameter between networks.

Conclusions: Capillary networks in the human perifovea are morphometrically heterogeneous. Morphometric features of regional capillary networks in the layered retina may serve a critical role in supporting neuronal homeostasis. Improved knowledge of these features may be important for understanding pathogenic mechanisms underlying retinal vascular diseases.

Introduction

The human retina has enormous capacity for parallel processing despite an average thickness of only 300 μm.[1] The capacity of the retina to partition light stimuli into a series of complex visual signals, prior to cortical transmission, is largely attributed to the layered organisation of neuronal populations.[2] Each neuronal population involved in visual processing has distinct metabolic demands with significant disparity in the rate of oxygen consumption between retinal layers.[3, 4] It is expected that the distribution of capillary networks within the retina correlate with the metabolic demands of soma, dendrites and synapses[5]– neuronal layers with the greatest energy demand may have the greatest capillary supply. Few studies however have aimed to validate this hypothesis.

Unlike other regions in the central nervous system, the retinal circulation must achieve neuronal nutrition without compromising the optical properties of the pathway transmitting light to the photoreceptor layer in the outer retina. Increases in neuronal energy demand cannot therefore be compensated by a simple increase in capillary number. It is likely that retinal capillary networks are morphometrically adapted in order that the balance between cellular nutrition and optical clarity can be achieved. In other organs, microcirculatory adaptive mechanisms that serve to satisfy the unique metabolic demands of distinct cell populations include modifications in capillary diameter,[6-14] inter-capillary distance,[6] capillary density[7, 11, 13-16] and the area bounded by capillary loops.[17] Similar capillary network adaptations may be present in the human retina.

Our laboratory has developed novel perfusion-based labelling techniques, utilising micropipette cannulation of the central retinal artery and targeted antibodies, to accurately label the human retinal circulation.[18-20] We have also coupled immunolabelling and microscopy techniques with this perfusion methodology to perform detailed analyses of

intercellular relationships in the human macula.[21] The present report employs previously validated state-of-the-art technology to quantify the morphometric features of capillary networks in a specialised region of the macula. The perifovea, located approximately 2 mm from the foveal centre, is examined.[22] This region is histologically characterised by approximately four rows of retinal ganglion cell nuclei and five to seven rows of nuclei in the inner and outer nuclear layers.[22] Nerve fibre layer (NFL) thickness in the perifoveal macula-papillary bundle is also greater than most other parts of the macula.[23] The retina is described to have one of the highest oxygen consumption rates of any tissue in the body and as a consequence is highly susceptible to irreversible injury during states of vascular compromise.[24, 25] The aim of this study is to improve our understanding of vascular mechanisms that support retinal homeostasis.

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

A total of 17 human eyes from 14 donors were used for this study. 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. The demographic data and medical co-morbidities of each donor are presented in Table 1.

Tissue Preparation

Our previously reported technique of central retinal artery cannulation, microvascular fixation and targeted endothelial cell labelling was used for this work.[19, 21] Briefly, the central retinal artery was cannulated using a glass micropipette and the retinal circulation perfused with a mixture of oxygenated Ringer's solution and 1% bovine serum albumin. After the 20-minute Ringer's wash the retinal circulation was perfusion-fixed using a solution of 4% paraformaldehyde in 0.1 M phosphate buffer. 0.1% Triton-X-100 in 0.1 M phosphate-buffered solution was then used to aid in the permeabilisation of endothelial cell membranes. Detergent was removed from the retinal circulation by perfusion with 0.1M phosphate buffer solution. Endothelial microfilaments and nuclei were labelled over 2 hours by perfusion with a solution comprising of phalloidin conjugated to Alexa Fluor 546 (30 U; A22283; Invitrogen, Carlsbad, CA) and bisbenzimide (H33258; 1.2 µg/mL; Sigma-Aldrich, St. Louis, MO). Residual label was cleared from the vasculature by further perfusion with 0.1 M phosphate buffer. Eye cups were immersion fixed in 4% paraformaldehyde overnight prior to dissection. The posterior globe was dissected at the equator to allow viewing of the posterior retina. Relaxing radial incisions were used to permit retinal flat mounting.

Immunolabelling

To aid co-localization between capillary networks and retinal layers further immunolabelling was performed in 4 post-perfused flat mount sections and 2 transverse retinal sections (Table 1). In 2 flat mount sections, the relationship between capillary networks, the nerve fibre layer and retinal ganglion cell layer (RGCL) was explored by incubating the retina with rabbit monoclonal γ-synuclein antibody[26] (Abcam ab55424, 1:200) followed by incubation with goat anti-rabbit antibody conjugated with Alexa Fluor 488 (Invitrogen A-11008, 1:200). In 2 flat mount sections, the relationship between capillary networks, bipolar cells and horizontal cells were explored using mouse-Goα antibody[27](Millipore MAB3073; 1:200) and rabbit Parvalbumin antibody[28] (Swant PV 25;1:500) respectively. Goat anti-mouse conjugated with

Alexa Fluor 488 and anti-rabbit antibodies conjugated with Alexa Fluor 633 were used for secondary labelling. All primary antibodies were incubated for 3 days followed by overnight incubation of secondary antibodies.[21]

Transverse retinal sections used for co-localization studies were of 12 μm thickness and were prepared from regions that were used for flat mount confocal microscopy studies. Transverse retinal sections were immunolabelled with γ-synuclein antibody[26] (Abcam ab55424, 1:200), Goα antibody[27] (Millipore MAB3073; 1:200) and parvalbumin antibody[28] (Swant PV 25;1:500). Lectin-TRITC[29](Sigma L5266, 1:40) was also used to label blood vessels in transverse sections.

Microscopy

In all flat mount retinal specimens confocal microscopy images were acquired from the peri-foveal region - located 2 mm nasal to the center of the fovea (Figure 1).[22] Wide-field images were captured prior to confocal microscopy with the aid of a x4 dry lens (Plan NA 0.2; Nikon, Tokyo, Japan) and a fluorescent microscope (Eclipse E800; Nikon). Wide-field images were used to accurately measure distances from the centre of the fovea prior to confocal scanning. Confocal microscope images were captured using a Nikon C1 Confocal with EZ-C1 (v. 3.20) image acquisition software. A x20 dry objective lens (NA 0.4) was used for all scans. Using a motorised stage, a series of Z stacks were captured for each specimen beginning from the vitreal surface, at the level of the inner limiting membrane, to the outer retina. Each z-stack consisted of a depth of optical sections collected at 0.35 μm increments along the z-plane.

Images of different wavelengths were acquired sequentially. Visualisation of sections labelled with Alexa Fluor® 408 secondary antibody was achieved by laser excitation at a 408 nm line from an argon laser with emissions detected through a 450/35 nm band pass filter.

Visualisation of sections labelled with Alexa Fluor® 488 secondary antibody was achieved by laser excitation at a 488 nm line from an argon laser with emissions detected through a 525/50 nm band pass filter. Visualisation of sections labelled with Alexa Fluor® 564 secondary antibody was achieved by laser excitation at a 561 nm line from an argon laser with emissions detected through a 595/40 nm band pass filter. Visualisation of sections labelled with Alexa Fluor® 637 secondary antibody was achieved by laser excitation at a 637 nm line from an argon laser with emissions detected through a 450/35 nm band pass filter.

Image Preparation

ImagePro Plus (Media Cybernetics, Version 7.1) and Image J (version 1.43, National Institute of Health, USA, http:/rsb.info.nih.gov/ij) were used to quantify confocal microscope images. All images for the manuscript were prepared using Adobe Photoshop (version 12.1, Adobe Systems Inc.) and Adobe Illustrator CS5 (version 12.1.0, Adobe Systems Inc.). Confocal images in this manuscript were pseudo-coloured using Look Up Tables available on ImageJ. Three-dimensional reconstruction of capillary network morphology was performed using the entire z-stack from a single donor (Table 1) and Imaris software (version 7.4.2, Bitplane, Zurich, Switzerland). To minimize artefact caused by minor fluctuations in signal intensity, the dataset for visualization was refined by utilizing the Gaussian filter tool and background subtraction prior to three-dimension reconstruction.

Qualitative Differentiation of Capillary Networks

Previous central nervous system studies have demonstrated that capillary networks within the brain, subserving distinct neuronal and glial populations, are characterised by unique cyto-architectural features.[12-14, 30] Morphometric criteria previously defined by these structure-

function histological studies were used to partition the retinal circulation into different capillary networks.

A z stack of confocal images that only contained information derived from the vascular channel of each eye was viewed using an animation sequence on Image J and a capillary network was defined as being different when one of the following morphometric criteria was satisfied:

- Alteration in projected direction and orientation of capillaries[12, 31, 32]
- Alteration in capillary branching pattern[12,31]
- Reduced presence of capillaries within retinal tissue

Qualitative division of the retinal circulation into different capillary networks was performed by 4 separate observers using the above criteria. Each observer recorded the location of the first and final image slice, within the z stack, for each capillary network. Inter-observer correlation between the number and location of capillary networks were subsequently performed (described below).

Following the division of z stacks into separate capillary networks, information acquired from remaining laser channels were merged with vascular channels and used to co-localize individual capillary networks with neuronal structures in the layered retina. Specifically, capillary networks were localized in relation to nuclei, the nerve fibre layer, ganglion cell layer, bipolar cells and horizontal cells. Quadruple-labelled transverse retinal sections were also used to aid in co-localization studies.

Morphometric Quantification of Capillary Networks

Quantitative data from capillary networks were attained following z projection of all images between the first and final image slice for each network as determined above. Capillary morphometric measurements were performed using previously defined histologic

parameters.[8, 14, 17] Manual tracing methods were used to attain the following quantitative measurements from each capillary network (Fig. 2):

- Capillary diameter - Defined as the perpendicular distance across the maximum chord axis of each vessel. Each confocal image was partitioned into 9 equal regions (Fig. 2B) and measurements were obtained from each region to ensure representative sampling. An average of 45 measurements was obtained from each image. Capillaries were defined as vessels with an absence of smooth muscle cells with diameter less than 10 μm.[12]
- Capillary loop area - Defined as the area circumscribed within visually enclosed capillary loops.[17]
- Capillary density - Defined as percentage of the sample area occupied by capillary lumens.

Quantification of Nuclei Density

Confocal images derived from the nuclear channel were used to quantify nuclei density. Projected z stacks, utilising the same confocal slices as that for capillary morphometric measurements, were used for nuclei counting. Nuclei density in each capillary network was calculated by employing previously reported manual thresholding techniques available on Image J software.[33] 'Analyze Particles' function on Image J software was utilised for nuclei counting and threshold parameters were based on the results of 100 manual nuclei measurements from random samples. Automated nuclei counts were determined for particles between 10 to 100 μm^2 and a circularity index between 0.20 to 1.00.

Statistical Analysis

All data is expressed in terms of mean and standard error which were calculated using Sigmastat (Sigmastat, ver. 3.1; SPSS, Chicago, IL). Multiple measurements from eyes with

data taken from right and left eyes of the same individual were analyzed using R (R Foundation for Statistical Computing, Vienna, Austria).[34] One-way analysis of variance (ANOVA) testing was performed to compare measurements between layers. The model used included "Right" or "Left" nested within "eye donor" as random effects using linear mixed modeling to test measurement differences between retinal layers.[34] The assignment of donor as a random effect was used to account for the effects of intra-"eye" correlation and similarly "Right" and "Left" to account for right and left eye correlation. ANOVA was also used to determine if "post-mortem time" (time to perfusion) influenced capillary morphometric measurements in retinal layers.

Inter-observer Correlation and Measurement Reproducibility

To assess inter-observer variation in distinguishing capillary networks, four masked observers were asked to identify stack numbers for the beginning and end of each morphometrically distinct capillary network in five eye specimens. Two-way ANOVA was performed modeling stack number with observer and layer to see if observer accounted for any variation in first or last stack number identified.

Twelve capillary microvasculature images derived from three different specimens were quantified on three separate occasions, each at least 1 week apart, by the same masked observer who performed all the data analyses. Capillary diameter, capillary loop area and capillary density were quantified for each image. Two-way ANOVA was used to assess the effect of day of measurement and capillary network layer on each of the capillary morphometric parameters with eye donor as a random effect. "Right" or "Left" was not considered because only one eye from each eye donor was used.

Results

Eye Donors

The mean age of donors was 50.71 ± 4.84 years (age range, 22 – 72 years). We examined 6 right eyes and 11 left eyes from a total of 13 male and 1 female donors. The average post-mortem time before eyes were perfused was 13.14 ± 3.51 hours.

General

All orders of retinal microvasculature were clearly labelled after perfusion labelling via the central retinal artery. Endothelial cells and nuclei were clearly identified following excitation with separate laser channels. Immersion immunolabelling of flat-mount and transverse retinal sections, post-perfusion, also allowed identification of capillaries relative to the NFL, RGCL, inner plexiform layer (IPL) and inner nuclear layer (INL). Transverse retinal sections (Fig. 3), from the region of interest, demonstrated a well delineated NFL, RGCL, IPL and INL. In the perifoveal region the RGCL demonstrated several strata of cell bodies (Fig. 1C).

Qualitative Study of Retinal Capillary Networks

Four morphologically varied retinal capillary networks were consistently observed by the four observers. Three-dimensional image reconstructions demonstrated the unique morphometric configuration of each network (Fig. 4). The morphometric features of each capillary network were as follows:

- *Nerve Fibre Layer (NFL) network* (Fig. 5) – Characterised by long capillary segments that were predominantly oriented parallel to the direction of retinal ganglion cell axons. A small number of shorter capillary segments, that interconnected long radial capillaries, were also seen in this network. Interconnecting capillaries were oriented either diagonal or orthogonal to long segments.
- *Retinal Ganglion Cell and Superficial Inner Plexiform Layer (RGCL/sIPL) network* (Fig. 6) – Characterised by a dense meshwork of three-dimensional vessels that were arranged in a lattice pattern with reduced inter-capillary spaces. Capillaries in this

network demonstrated looping hairpin turns that projected vertically. There was also close approximation between RGCL capillaries and the larger retinal vessels.

- *Deep Inner Plexiform and Superficial Inner Nuclear Layer (dIPL/sINL) network* (Fig. 7) – Capillaries in this network comprised of vertical and oblique segments that resulted in an irregularly shaped loop configuration. Capillaries in this network demonstrated great tortuosity.
- *Deep Inner Nuclear Layer (dINL) Network* (Fig. 8) – Capillaries in this network were arranged in a one-dimensional laminar configuration. Capillaries ran a linear trajectory with little tortuosity.

Quantitative Characteristics of the Nerve Fibre Layer Network

Mean capillary diameter in the NFL network was 8.30 ± 0.07 μm (n = 347). A total of 84 capillary loops were measured with a mean area of 4940.31 ± 529.38 μm^2. Capillary density in the NFL network was 17.37 ± 0.99%. NFL capillary measurements from individual donors are presented in Table 2. Mean nuclei density in the NFL network was 1552.89 ± 109.64 cells per mm^2.

Quantitative Characteristics of the Retinal Ganglion Cell and Superficial Inner Plexiform Layer Network

Mean capillary diameter in the RGCL/sIPL network was 8.29 ± 0.07 μm (n = 304). A total of 102 capillary loops were measured with a mean area of 3954.04 ± 505.30 μm^2. Capillary density in the RGCL/sIPL network was 22.32 ± 0.99%. RGCL/sIPL capillary measurements from individual donors are presented in Table 2. Mean nuclei density in the RGCL/sIPL network was 2201.13 ± 113.70 cells per mm^2.

Quantitative Characteristics of the Deep Inner Plexiform and Superficial Inner Nuclear Layer Network

Mean capillary diameter in the dIPL/sINL network was 8.25 ± 0.07 μm (n = 325). A total of 63 capillary loops were measured with a mean area of 5424.03 ± 602.72 μm^2. Capillary density in the dIPL/sINL network was 19.56 ± 0.99%. dIPL/sINL network capillary measurements from individual donors are presented in Table 2. Mean nuclei density in the dIPL/sINL network was 3051.47 ± 168.71 cells per mm^2.

Quantitative Characteristics of the Deep Inner Nuclear Layer Network

Mean capillary diameter in the dINL network was 8.26 ± 0.07 μm (n = 372). A total of 66 capillary loops were measured with a mean area of 6866.52 ± 584.58 μm^2. Capillary density in the dINL was 17.95 ± 0.99%. dINL capillary measurements from individual donors are presented in Table 2. Mean nuclei density in the dINL network 3792.59 ± 235.09 cells per mm^2.

Morphometric Comparisons Between Networks

Post-mortem time was not associated with capillary diameter, loop area or density measurements in any of the networks (all $P > 0.142$). There was no difference in capillary diameter between networks ($P = 0.715$). Capillary loop area was smallest in the RGCL/sIPL network and was significantly smaller than the dIPL/sINL network ($P = 0.028$) and dINL network ($P < 0.003$) but not the NFL network ($P > 0.050$). There was no difference in capillary loop area between dIPL/sINL and dINL networks ($P = 0.088$) and also between NFL and dIPL/sINL ($P = 0.632$) and dINL networks ($P = 0.107$).

Capillary density was greatest in the RGCL/sIPL network and was significantly greater than the NFL network ($P = 0.015$) and dINL ($P = 0.004$). There was no difference in capillary

density between RGCL/sIPL network and dIPL/sINL networks ($P = 0.074$), dIPL/sINL and dINL ($P = 0.069$), NFL and dIPL/sINL ($P = 0.179$) and also NFL and dINL networks ($P = 0.689$).

Mean nuclei density increased with progression through the NFL, RGCL/sIPL, dIPL/sINL and the dINL networks when layer was treated as a continuous variable *(*$P = 0.000$). Nuclei density was lowest in the NFL network and was significantly lower than the dIPL/sINL network ($P = 0.013$) and dINL network ($P = 0.009$). Nuclei density was greatest in the dINL network and was significantly greater than RGCL/sIPL network ($P = 0.010$). There was no difference in nuclei density between NFL and RGCL/sIPL network ($P = 0.051$) and dIPL/sINL and dINL network ($P = 0.062$).

Inter-observer Correlation and Measurement Reproducibility

The assignment of first and last stack numbers, to define capillary networks, was not different between the four observers ($P = 0.999$). Analysis of measurement reproducibility did not reveal a significant difference in capillary diameter measurement between the 3 measurement days ($P = 0.836$). There was also no significant difference between the 3 measurement days for capillary loop area ($P = 0.976$) and capillary density measurements ($P = 0.549$).

Discussion

The major findings from this study are: (1) Capillary networks in the human perifovea demonstrate morphometric variation according to retinal layer. (2) Capillary density varies between retinal layers and is greatest in the RGCL/sIPL and dIPL/sINL networks. (3) Capillary loop area is smallest in the two innermost networks. (4) There is no difference in capillary diameter between the layers of the retina.

The perifovea is a specialised region of the human eye that is histologically and functionally distinct in comparison to other portions of the retina.[22] Unlike the peripheral retina, which is comprised of only a single row of ganglion cells, the nasal perifovea is characterised by 4-5 rows of retinal ganglion cells.[22] Additionally, the nerve fibre layer in the perifovea is thicker than in other parts of the macula.[23] The macula-papillary bundle traverses the perifovea and is the conduit through which a large quantity of pre-cortically processed retinal information is transmitted to the brain. Consequently, diseases that preferentially affect the macula-papillary bundle result in devastating visual morbidity.[35] Understanding the structure of capillary networks serving the perifovea may provide insights into vascular-mediated mechanisms that satisfy the metabolic demands of this region.

The present study identified four morphometrically different capillary networks within the human perifovea. This finding was verified by four masked, independent observers. The innermost and outermost networks, situated in the NFL and deep portion of the INL respectively, demonstrated a laminar, one-dimensional configuration. Capillary networks in the NFL were also observed to project parallel to the trajectory of retinal ganglion cell axons and resembled the microcirculation described in skeletal muscle[36] where capillaries are oriented parallel to the direction of muscle fibres. Interconnecting, orthogonally-oriented anastamoses are also seen in the NFL, similar to skeletal muscle capillary systems.[36] In contrast, the capillary networks located in the RGCL/sIPL and dIPL/sINL demonstrated a tortuous, three-dimensional architecture that resembled the voroni tessellation described in cortical capillary beds.[37] The variation in retinal capillary network morphology identified in the present study demonstrates important parallels to the human cerebral cortex where the microcirculation is also altered according to neuronal layer.[38-40]

Unlike the brain, the retina is readily accessible for investigating the physiological behaviour of subcellular components within distinct neuronal layers. Using oxygen sensitive microelectrode techniques, intra-retinal oxygen distribution and oxygen uptake in different cellular layers has been quantified during physiological and non-physiological states.[4, 25, 41-48] These previous studies have identified three distinct regions of high oxygen uptake: (1) The inner segment of photoreceptors. (2) The inner plexiform layer. (3) The outer plexiform layer.[3, 25, 49] The relationship, and proximity, between each region of high oxygen uptake and the local microcirculation however is vastly different. Detailed studies have shown that photoreceptors, including their nuclei, the high energy-consuming inner segments and the photosensitive outer segments, lie within the avascular layers of the retina.[3] Oxygen and nutrient supplies to photoreceptors are completely dependent upon diffusion mechanisms from choroidal and deep retinal vascular beds.[50] Although oxygen tension in the choroid is high, oxygen tension at the level of the inner segments is paradoxically low.[3] In contrast, the inner half of the retina is supported by a sparse distribution of retinal vessels with significant disparities in oxygen levels between inner retinal layers.[3] The present study identifies important relationships between neuronal sub-compartments and regional capillary network morphometry and may be important for understanding vascular-mediated mechanisms that account for the heterogeneous oxygen profile across the inner retina. It may also identify vascular-mediated mechanisms that permit momentary variations in neuronal metabolic demands to be satisfied.[25, 44] Retinal glia are likely to play a critical role in modulating changes in regional blood supply consequent to variations in neuronal demands.[51]

Investigating how retinal capillary network topography is coupled with regional neuronal demands is important for understanding physiological mechanisms that support retinal homeostasis. The present study demonstrates that the inner plexiform layer is supported by two capillary networks - situated in the inner and outer boundaries of the IPL. Capillary

density is greatest in these networks, relative to other retinal layers, suggesting that the energy demands of neuronal arborisations are high. The three-dimensional organisations of these networks most likely serve to increase oxygen delivery and waste removal within the IPL.[30, 52] It was surprising that the central portion of the IPL was relatively devoid of vasculature, however this region is known to have considerable Muller cell support.[53] There is increasing evidence to suggest that glial cells play a key role in modulating regional blood supply via neurotransmitter-mediated signalling – particularly through the release of glutamate.[54] Glutamate-mediated signalling leads to the release of arachidonic acid derivatives from glial cells and nitric oxide molecules from neurons with the net effect being an increase or decrease in blood flow, depending on local oxygen concentration.[54] Through these signalling mechanisms it is possible for Muller cells to control regional oxygen distribution in different capillary networks. Additionally, Muller cells support the metabolic activity of regional neurons through metabolic symbiosis, a neuron-muller interaction where pyruvate released by Muller cells is used as a substrate by neighbouring neurons to generate energy through Krebs cycle.[53] We speculate that the latter mechanism is an important means by which Muller cells support neuronal metabolic activity in regions with scant capillary supply, such as the mid-portion of the IPL. Further work however is required to validate this hypothesis.

Inter-capillary areas bear important relationships to oxygen diffusion properties and it is postulated that decreasing inter-capillary areas result in decreased oxygen diffusion times[17] – the net effect being increased ATP production. Capillary loop area was lowest in NFL and RGCL/sIPL networks suggesting that oxygen diffusion may be an important mechanism by which neuronal function is supported in these layers. The significant differences in capillary loop area between the two networks that serve the IPL also suggests that the process of oxygen diffusion plays a disparate role in supporting the inner and outer portions of the IPL.

Detailed histological studies have revealed that the vertebrate IPL is a non-uniform, layered structure with significant dissimilarities in the density and complexity of synaptic contacts between IPL strata.[55-57] Furthermore, there is significant heterogeneity in the ratio of amacrine:bipolar:ganglion cell synapses between IPL strata. The axon terminals of ON- and OFF-bipolar cells ramify in distinct IPL strata with terminal arborizations of OFF-type and ON-type cells synapsing in sublamina *a* and *b* of the IPL respectively.[58] We speculate that the heterogeneous metabolic demands of distinct IPL strata, and the unique role served by each strata in parallel processing, may account for the differences in capillary network morphometry between superficial and deep IPL capillary networks. Differences in capillary loop areas between networks may be one means by which the distinct metabolic demands of inner and outer IPL strata are satisfied. Mean capillary diameter is also known to influence the rate of capillary oxygen exchange.[59] Unlike studies in intra-cortical capillary networks[60] we did not detect significant differences between perifoveal capillary network diameters.

The purpose of this study was to identify major differences in capillary network morphometry between perifoveal layers. Our experimental model of central artery cannulation and perfusion is best suited for such an investigation as it ensures reliable and complete labelling of the retinal microcirculation. Confocal microscopy and immunohistochemical techniques developed in our laboratory also ensured accurate correlation of capillary-neuronal relationships. Trypsin digestion[61] and vascular casting[62] techniques were previously used to study the retinal microcirculation however inadvertent tissue destruction consequent of these methodologies limited accurate delineation of such inter-relationships. Although our experimental methodology allowed us to precisely control and monitor perfusion pressure in the retinal circulation, the effect of post-mortem artefact may still have influenced some of the morphometric measurements in our study. We accounted for the effect of post-mortem artefact in our statistical analysis and we did not find

that this variable was associated with capillary density, loop area or diameter measurements in any of the retinal layers. Our results are consistent with previous studies involving cerebral capillaries where a significant change in post-mortem capillary diameter was not demonstrated in human or cat cortical tissue.[63] However, mean capillary diameters in the present report are larger than other regions of the central nervous system[8] and this may be consequent to contractile mechanisms that continue to act on vasculature in the acute period after death.[64] Cerebral artery diameters in humans and monkeys are known to respond to extracellular milieu changes in the first 24 hours post-mortem.[64] Similar mechanisms may continue to act on retinal capillary networks in the immediate post-mortem period and thereby influence capillary diameter measurements reported in the present study. However, the effects of these mechanisms are expected to be equal in all networks thus permitting useful inter-network comparisons to be performed during this immediate post-mortem period.

The results of this study suggest that capillary network morphometry is coupled with neuronal demands in the human perifovea. It also demonstrates that the correlation between neuronal metabolic activity and capillary network location is not exact. The IPL is supported by capillary networks that are situated on the boundaries of this layer and not within it. The mismatch between capillary network location, morphometry and neuronal activity in different layers demonstrates a degree of dissimilarity between retinal capillary networks and the microcirculation in other regions of the central nervous system. In the brain, there is a strong correlation between capillary density and the metabolic requirements of layered neuronal structures.[8, 40, 60] The findings in the present study also improve our understanding of the relationships between capillary organisation and intra-retinal oxygen distribution and uptake in the human retina.[3] Oxygen tension in the photoreceptor layer, a predominantly avascular region, is exceedingly high.[65] Similarly, sub-compartments in the inner retina which do not have high density capillary networks are also capable of maintaining high oxygen levels.

These findings implicate a vital role served by non-vascular structures, such as Muller cells and glia, in nourishing neuronal populations and controlling retinal homeostasis. The differences in neuron-capillary relationships between the retina and brain may be due to the following reasons: (1) The organisation of vascular structures in the retina are constrained by the optical properties of the eye. (2) The distribution and organisation of Muller cells and astrocytes in the retina are different to the brain. (3) The cellular inter-relationships in the retina are arguably more complex than the brain. Improved understanding of neuron-vascular-glial relationships in the human retina will enhance our understanding of patho-physiological processes involved in retinal vascular diseases.

Capillary network morphometry is altered with disease[8] and it will therefore be important to perform similar morphometric studies using abnormal human eyes. A small foveal avascular zone is known to persist in preterm infants despite the absence of clinically evident retinopathy of prematurity (ROP).[66] In some patients with oculocutaneous albinism the central macular area is also crossed by capillaries.[67] The findings from these previous studies suggest that visual acuity changes in patients with ROP and albinism may be partly due to abnormal macular capillary networks. It is also likely that the presence of capillary structures in retinal eccentricities that are normally devoid of vasculature result in altered neuronal homeostasis and optical clarity which in-turn may adversely affect retinal function. Further studies are required to delineate the pathogenic mechanisms through which altered retinal capillary networks affect visual acuity particularly in patients with blunted foveal depressions and small foveal avascular zones.

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Legends

Figure 1 – Human perifovea. Colour fundus (A) and corresponding Zeiss Cirrus high-definition ocular coherence tomography image (B) from a healthy subject illustrate the perifoveal region that was used for capillary morphometric studies (fenestrated box). Nerve fibre layer thickness in the perifoveal region (indicated by arrows) is greater than most other parts of the macula. Toluidine blue stained section from a separate subject demonstrates normal perifoveal histology (C). This region has approximately four rows of retinal ganglion cell nuclei and five to seven rows of nuclei in the inner and outer nuclear layers. OD = Optic Disk, NFL = Nerve Fibre Layer, RGCL = Retinal Ganglion Cell Layer, IPL = Inner Plexiform Layer and INL = Inner Nuclear Layer. Scale bar in colour fundus and ocular coherence tomography image = 1000 μm. Scale bar in toluidine blue histology = 50 μm.

Figure 2 – Methodology for quantification of capillary network morphometry. Representative Z-projected confocal microscope image of the deep inner nuclear layer network (A) and a corresponding manually traced image (B) illustrate the vascular parameters that were measured. Vessel density (highlighted in red) was expressed as a percentage of total area. Capillary loop area (highlighted in green) expressed as μm^2 and capillary diameter (blue marks) expressed as μm were also measured. Each image was divided into 9 equal portions (fenestrated lines) and capillary diameter measurements were obtained from each region. Scale bar = 100 μm.

Figure 3 - Co-localization of capillary networks within retinal layers. Triple- (A) and quadruple-labelled (B) transverse retinal sections demonstrate the relationship between capillary networks and nerve fibre layer (NFL), retinal ganglion cell layer (RGCL), inner plexiform layer (IPL) and inner nuclear layer (INL). Endothelial cells are labelled with Phalloidin, nuclei are labelled with Hoescht, RGCL and NFL are labelled with γ-synuclein. Goα and Parvalbumin were used to label Bipolar and Horizontal cells, respectively. Four

capillary networks (fenestrated red brackets) were identified in the inner retina. The innermost capillary network was identified in the NFL. The second network was located at the level of the RGCL and superficial portion of the IPL. A separate network was identified at the boundary between the deep portion of IPL and superficial portion of INL. The outermost capillary network was identified at the level of the deep INL. Scale bar = 50 μm.

Figure 4 – Three-dimensional morphometry of human perifoveal capillary networks. Contour surface-rendered images generated using Imaris software demonstrates the complex organization of different capillary networks in the human retina from two different angles of reconstruction (A) and (B). Arrow allows retinal orientation and indicates projection commencing at the vitreal surface and extending to the outer retina. Scale bar = 150 μm.

Figure 5 – Nerve Fibre layer capillary network. Endothelial cells (labelled with phalloidin) stain red, nuclei (labelled with Hoescht) stain blue and NFL axons (labelled with Anti-γ-synuclein) stain green. Confocal capillary images captured from a single laser channel (A) demonstrate that the trajectory of a large number of capillaries parallel axons in the nerve fibre layer. Merged images (B) demonstrate a relative paucity of nuclei within this network compared to other retinal layers examined. Capillary images from a separate donor (C) merged with a stain specific for retinal ganglion cell axons (D) demonstrate co-localization of this network with the nerve fibre layer of the retina. Scale bar = 100 μm.

Figure 6 – Ganglion Cell layer/superficial Inner Nuclear layer network. Endothelial cells (labelled with phalloidin) stain red, nuclei (labelled with Hoescht) stain blue and retinal ganglion cells (labelled with Anti-γ-synuclein) stain green. Confocal capillary images captured from a single laser channel (A) demonstrate the complex capillary configuration in this network. The proximity of capillaries to larger order arterioles and veins in this network is also demonstrated. Merged images (B) demonstrate a high concentration of nuclei within this network. Capillary images from a separate donor (C) merged with a stain specific for

retinal ganglion cell nuclei (D) demonstrate co-localization of this network with the ganglion cell layer of the retina. Scale bar = 100 μm.

Figure 7 – Deep Inner Plexiform layer/superficial Inner Nuclear layer network. Endothelial cells (labelled with phalloidin) stain red, nuclei (labelled with Hoescht) stain blue, bipolar cells and their cell processes (labelled with Goα) stain green. Confocal capillary images captured from a single laser channel (A) demonstrate the irregularly shaped loop configuration of capillaries in this network. Merged images (B) demonstrate a high concentration of nuclei within this network. Capillary images from a separate donor (C) merged with bipolar cell markers (D) demonstrate the intimate relationship of capillaries to processes of bipolar cells which co-localize this network with the inner plexiform layer of the retina. Scale bar = 100 μm.

Figure 8 – Deep Inner Nuclear layer network. Endothelial cells (labelled with phalloidin) stain red, nuclei (labelled with Hoescht) stain blue, bipolar cells (labelled with Goα) stain green. Confocal capillary images captured from a single laser channel (A) demonstrate the planar configuration of capillaries in this network. Merged images (B) demonstrate a high concentration of nuclei in the region of this network. Capillary images from a separate donor (C) merged with Bipolar cell markers (D) demonstrate a similar concentration of nuclei in comparison to the superficial INL network. Scale bar = 100 μm.

Patient ID	Sex	Age	Eye	Co-morbid conditions	Time to perfusion (hrs)
A	M	32	L	MVA	20
B	M	23	L	Suicide	22
C	M	53	L	MVA	14
D‡	M	66	R+L	Colon Cancer	15
E	M	22	L	Suicide	15
F	M	27	L	MVA	8
G	M	59	R+L	Melanoma	12
H	M	39	L	Bacterial Endocarditis	20
I	M	68	R	COPD	11
J#	M	60	L	Prostate Cancer	18
K#	M	65	R	Cardiomyopathy	6.5
L#	F	64	L	Short illness	5
M#	M	72	R	Drowning	15
N▲	M	60	R+L	Alzheimers Disease	2.5

Table 1 – Donor demographic details. Age (years), sex (M = male or F = female), cause of death and time to perfusion for each eye donor is provided. Donor eyes that were flat mounted for co-localisation studies are designated (#) and those that were sectioned transversely for co-localisation studies are designated (▲). Confocal data from one donor was used for three-dimensional capillary reconstruction (‡). MVA = motor vehicle accident, COPD = chronic obstructive pulmonary disease.

Specimen	Capillary diameter (μm)				Capillary loop area (μm^2)				Capillary density (%)			
	NFL	*RGCL/sIPL*	*dIPL/sINL*	*dINL*	*NFL*	*RGCL/sIPL*	*dIPL/sINL*	*dINL*	*NFL*	*RGCL/sIPL*	*dIPL/sINL*	*dINL*
A	8.6	8.1	8.5	7.6	2462.5	4823.8	9397.2	15593.1	15.0	23.4	16.5	14.7
B	8.1	7.8	6.5	7.4	7494.5	1814.7	0.0	4841.8	14.8	25.3	14.4	18.9
C	8.3	8.8	8.8	9.0	4684.0	8754.2	3436.5	4169.8	18.6	16.8	20.4	17.6
D	8.4	8.3	8.7	9.2	5394.9	2826.6	7461.2	6307.3	22.4	26.0	24.6	24.2
D	8.8	8.0	8.3	7.5	6987.6	2782.1	2354.3	4562.3	20.2	34.6	25.0	21.7
E	8.6	8.8	8.1	8.1	5959.9	6000.8	8412.4	7158.17	22.0	16.2	14.1	15.2
F	7.9	8.3	8.4	8.9	3543.7	5318.5	5401.9	7326.5	10.5	21.1	20.7	18.9
G	7.9	8.3	8.2	8.5	5688.8	0.0	9063.6	9813.8	19.2	19.6	19.7	15.2
H	8.7	8.6	8.1	8.4	3789.6	6234.2	4472.2	0.0	17.4	16.8	15.0	12.6
I	7.6	8.3	8.8	8.4	1645.9	5622.6	3219.5	4371.5	14.8	21.2	23.1	21.3
J	8.2	7.3	8.4	8.0	4788.9	3500.3	3979.8	8799.2	16.3	24.6	21.7	17.2

Table 2 – Quantitative capillary network data for individual donors. Mean capillary diameter, capillary loop area and capillary density measurements for each donor eye is provided. NFL = Nerve Fibre Layer, RGCL = Retinal Ganglion Cell Layer, sIPL = superficial Inner Plexiform Layer, dIPL = deep Inner Plexiform Layer, sINL = superficial Inner Nuclear Layer and dINL = deep Inner Nuclear Layer.

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