

Quantifying cortical tissue and vascular properties using optical coherence tomography

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This dissertation by Sabina Stefan is accepted in its present form by the Biomedical Engineering graduate program as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract of “Quantifying cortical tissue and vascular properties using optical coherence tomography” by Sabina Stefan, Ph.D., Brown University, May 2022.

Optical coherence tomography (OCT) is becoming increasingly popular for neuroscientific study, but it remains challenging to objectively quantify tissue and vascular properties from 3D or 4D OCT data. For tissue imaging, OCT provides structural imaging capability, which can be utilized for the determination of the attenuation coefficient. However, the accuracy of the attenuation coefficient is dependent on the precise characterization of the confocal effect and the position of the focal plane within the sample, which may be tilted or curved. This thesis presents a method to accurately determine the 2D position of the focal plane within the sample, which is also applicable to high-magnification data. We demonstrate applicability to real biological data of glioblastoma acquired *in vivo* in a murine model.

For vascular imaging, OCT angiography can be performed to visualize perfused vessels, and capillary red blood cell (RBC) flux can also be quantified by acquiring time-series data at each 3D position. However, it remains challenging to quantify angioarchitectural properties from 3D angiograms, mainly due to projection artifacts or tails underneath vessels caused by multiple-scattering, as well as the relatively low signal-to-noise ratio compared to fluorescence-based imaging modalities. Challenges also exist in quantifying RBC flux which typically relies on peak-counting of transient peaks in intensity associated with RBC passages, and is dependent on user parameterization. To overcome these challenges, we leverage deep learning to enhance, segment and connect vessels in OCT angiograms, as well as to predict RBC flux from time-series data. These tools enable the extraction of the underlying 3D vascular graph and the subsequent quantification of vascular properties, as well as the estimation of RBC flux across an entire network of hundreds of capillaries.

Finally, we leveraged these tools to track vascular changes in the same set of animals over a period of 7 months. These integrated methods enabled us to simultaneously monitor 24 distinct vascular properties spanning morphology, topology, and function of the microvasculature across all scales: large pial vessels, penetrating vessels, and capillaries. We demonstrate the efficacy of this approach in normal mice, and differential onset of vascular changes in mice that model mechanisms of Alzheimer's disease. This new approach will allow comprehensive and longitudinal study of a broad range of progressive vascular diseases, and normal aging, in key model systems.

Curriculum Vitae

Education

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- 2022 **Stefan, S.**, Kim, A., Marchand, P. J., Lesage, F., & Lee, J. Deep learning and simulation for the estimation of red blood cell flux with optical coherence tomography. *Frontiers in Neuroscience*, 64.
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- 2018 **Stefan, S.**, Jeong, K. S., Polucha, C., Tapinos, N., Toms, S. A., & Lee, J. (2018). Determination of confocal profile and curved focal plane for OCT mapping of the attenuation coefficient. *Biomedical optics express*, 9(10), 5084-5099.
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- 2020 **Stefan, S.** & Lee, J. Deep Learning Toolbox for Vectorizing Microangiograms, Brown Unconference on Computational Intelligence and Applications
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1. Introduction

1.1. Motivation

The brain is a complex system, which humans have tried to study and understand for thousands of years. Over time, we have developed increasingly sophisticated tools to probe this complex organ to finesse our knowledge and gain new insights. Nowadays, there are a myriad of tools at our disposal: animal models, imaging technologies, surgical preparations, staining protocols, electrophysiology, fluorescent markers, and so forth. With each new technology that is developed, there is the possibility of testing new hypotheses which were not previously possible. In other words, there is the possibility of pushing the envelope forward (even just a little) on how the brain functions in health, as well as how it dysfunctions in disease.

One important tool for probing the brain is imaging, which can be accomplished using a number of different methods and at different scales. Macroscale imaging techniques include functional magnetic resonance imaging (fMRI) and positron emission tomography (PET). Based on blood flow, fMRI has been used extensively to study the neural activity underlying cognitive function related to language [1], sensory processing [2], and memory [3]. In addition to these fundamental questions, fMRI has also shown promise in diagnosing neurodegenerative disease [4] and guiding surgical care of brain cancer patients [5]. Likewise, PET can be used to detect the earliest signs of Alzheimer's disease by using tracers which detect amyloid-beta in the brain [6], a potential biomarker for Alzheimer's disease. While such imaging techniques provide structural and functional insight pertinent to the whole brain, micro-level resolution is not yet feasible. Instead, other in-vivo imaging technologies, such as multi-photon microscopy (MPM),

combined with rodent models have become popular for the testing of hypotheses requiring greater granularity.

MPM has already demonstrated its usefulness in characterizing brain cell structure [7], neuronal signaling [8], blood flow regulation [9] and metabolism [10]. However, several drawbacks still exist: exogenous fluorescent labeling is often required, photobleaching and/or phototoxicity may occur, and imaging of deep tissue may be challenging due to the degradation of signal [11,12]. Optical coherence tomography, which is already widely-adopted in ophthalmology, is quickly gaining traction in neuroscience thanks to the unique advantages that it offers. It is a minimally invasive imaging technique which can rapidly produce 3D images without the need for a contrast agent, with superior imaging depth and field of view as compared to two-photon microscopy. OCT has already shown promise in the visualization of neurons (and their myelinated axons) [13] and cortical vessels [14], as well the quantification of blood flow [15].

However, there is still a wealth of information that could be extracted from OCT data, along with questions about how best to quantify this information in a manner that most accurately reflects the underlying physiology. Computational tools are an integral part of this process, enabling both objectivity and automation: objectivity is critical for comparisons between experimental groups or even between different studies, and automation is essential for making the analysis of large image sets both realistic and unbiased. Towards this goal, this thesis is concerned with the quantification of OCT data in four central topics: the optical properties of cortical tissue, the structure of the microvasculature, blood flow within capillaries, and the longitudinal tracking of vascular features.

1.2. Chapter overview

This thesis is divided into 6 chapters. Chapter 1 gives a brief overview of OCT and its working principles. The physics and mathematical principles underlying this technology are briefly explained, and the functionality of OCT for structural and functional imaging of the cortical vasculature is explored. Some examples are presented on how OCT can be leveraged for angiography and blood flow quantification in both penetrating vessels and capillaries. Chapter 2 is focussed on quantifying structural OCT data by the accurate determination of the attenuation coefficient. This is based on the extraction of the 2D focal plane, which may be slanted or curved within the sample, which is especially critical for high-NA lenses (or equivalently, high-magnification imaging). The method is demonstrated on glioblastoma in a murine model. Chapter 3 is concerned with the quantification of vascular properties from 3D microangiograms. This is a challenging task due to the presence of projection artifacts or tails in the axial dimension due to the presence of multiple-scattering, as well as the relatively low signal-to-noise ratio compared to other popular microscopic vascular imaging modalities, such as multi-photon microscopy. Chapter 4 describes progress toward blood flow quantification in a network of capillaries, in which RBCs flow in single-file. The proposed method utilizes two-photon microscopy and OCT data which were acquired simultaneously to train a convolutional neural network to predict RBC flux. This CNN is further refined by simulated RBC passages, which were simulated using insights from the experimental data. Chapter 5 demonstrates how several OCT imaging capabilities can be combined to longitudinally track changes in the vasculature of Alzheimer's disease model mice as compared to wild-type mice. Lastly, Chapter 6 gives an overview of the work accomplished in this thesis and future directions.

1.3. The theory of OCT

Optical coherence tomography is a 3D, label-free imaging modality based on the backscattering of light with the capability of producing high resolution images. The axial resolution ranges from 1-15 μm and is dependent on the bandwidth of the light source, whereas the lateral resolution is dependent only on the choice of objective lens. In most biological samples, a penetration depth of 1-3 mm can be achieved, mainly depending on scattering coefficient. OCT is based on the classical principle of low-coherence interferometry, which describes the interference of back-scattered light from the tissue with light that has traveled along a reference path with known time delay. This is accomplished by splitting the incident light source into a reference beam $E_R(t)$ and a signal beam $E_S(t)$, which eventually are recombined creating an interference pattern at the detector (see Fig. 1.1).

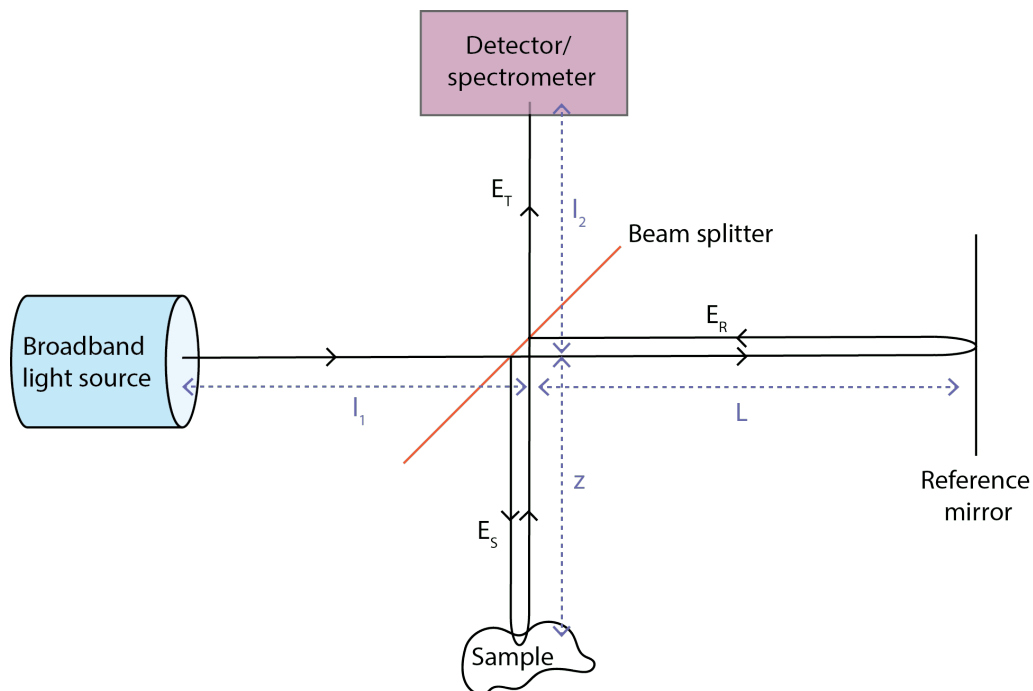


Figure 1.1. Simplified OCT setup.

For a monochromatic light source, the magnitude of the interference fringes is periodic as a function of the difference in path length between the sample and reference beams. This makes it impossible to determine depth information with a continuous monochromatic light source, necessitating a low-coherence broadband source. A broadband source describes a light source which is a superposition of many frequency components. With such a light source, interference occurs when the reference and sample beams are within a coherence length of the light source, allowing for depth-resolved imaging where the signal is strongest when the sample and reference arms are matched. For a Gaussian source spectrum, the coherence length is defined as,

$$l_c = \frac{4 \ln 2}{\pi} \frac{\lambda_0^2}{\Delta \lambda} \quad (1.1)$$

where l_c is the coherence length, λ_0 is the center wavelength and $\Delta \lambda$ is the full width at half maximum (FWHM) of the bandwidth of the light source.

The first generation of OCT is time-domain OCT (TD-OCT), in which the reference mirror is scanned over a given length to vary the path lengths between the sample and reference beams, thereby allowing for the sample reflectivity to be obtained as a function of depth. To be more precise, the intensity of at the detector can be determined by summing the sample beam, $E_S(t, \omega)$, and reference beam defined as, $E_R(t, \omega)$, which in this case are divided 50-50 by the beam splitter,

$$E_S(t, \omega) = \frac{1}{2} \tilde{R} E_0(\omega) e^{ik(\omega)(2z+l_1+l_2)-i\omega t} \quad (1.2)$$

$$E_R(t, \omega) = \frac{1}{2} E_0(\omega) e^{ik(\omega)(2L+l_1+l_2)-i\omega t} \quad (1.3)$$

where distances l_1, l_2, z and L are demonstrated in Fig. 1.2, $\tilde{R}$ is the complex reflectivity of the point sample, k is the wavenumber which is a function of the angular frequency ω , and $E_0(\omega)$ is the broadband light source. The total complex light at the detector is then,

$$\widetilde{E}_T(t, \omega) = \frac{1}{2} E_0 (1 + \tilde{R} e^{i2k(\omega)(z-L)}) e^{ik(\omega)(2L+l_1+l_2)} e^{-i\omega t} \quad (1.4)$$

From which the intensity can be calculated as,

$$I(\omega) = \frac{1}{2} \epsilon_0 c |\widetilde{E}(t, \omega)|^2 = \frac{1}{8} \epsilon_0 c E_0^2(\omega) (1 + |\tilde{R}|^2 + \tilde{R} e^{i2k(\omega)(z-L)} + \tilde{R}^* e^{-i2k(\omega)(z-L)}) \quad (1.5)$$

The total intensity at the detector can then be determined by integrating over ω . Assuming that

$k(\omega) = \frac{\omega}{c}$, and that $E_0^2(\omega)$ is Gaussian, the intensity is,

$$I = \frac{1}{16\pi} \epsilon_0 c E_0^2 (1 + |\tilde{R}|^2 + 2|\tilde{R}| \exp(-\frac{2\sigma_\omega^2}{c^2}(z-L)^2)) \cos(2k_0(z-L)) \quad (1.6)$$

Where k_0 is the central wavelength, and σ_ω is the standard deviation of the Gaussian light source. The envelope which modulates the cosine term in Eq. 1.6 can be considered a point spread function, which allows for depth imaging by adapting L (i.e., moving the reference mirror).

However, the axial scanning of the reference mirror limits the acquisition speed, which is circumvented by the later innovation of the spectral domain OCT (SD-OCT). Unlike TD-OCT, SD-OCT does not require movement of the reference beam, allowing imaging speeds which are up to ~100x faster. This is achieved by replacing the point detector with a spectrometer which collects backscattered light from all the depths, and performing a Fourier transform from the wavenumber domain to the spatial domain to obtain depth information. The intensity spectrum at the spectrometer as a function of the wavenumber, k , can be expressed as,

$$I(k) = \frac{1}{8} \epsilon_0 c E_0^2(k) (1 + |\tilde{R}|^2 + \tilde{R} e^{i2k(z-L)} + \tilde{R}^* e^{-i2k(z-L)}) \quad (1.7)$$

or equivalently,

$$I(k) = \frac{1}{8} \epsilon_0 c E_0^2(k) (1 + |\tilde{R}|^2 + 2|\tilde{R}| \cos(2k(z-L) + \phi_R)) \quad (1.8)$$

where ϕ_R is a phase term. Assessing this intensity as a function of k , we find that the first term is dependent only on the source spectrum, the second term is similarly a constant multiplied by the source spectrum (in the case of a point sample), while the third term contains the

information pertaining to depth. More specifically, the third term of Eq. 1.8 shows that small path differences (i.e small $(z - L)$) result in small frequency modulations, and vice versa for large path differences. This is the key which allows the determination of depth information. For practical purposes, post-processing of the intensity in Eq. 1.8 can include either subtraction or division of the first term, representing the source spectrum, before obtaining the inverse Fourier transform. Furthermore, while the equations describe a point sample, they can easily be extended to apply to a continuous sample with reflectivity as a function of depth, $\tilde{R}(z)$. This can be accomplished by updating Eq. 1.2 which describes the light which is reflected from the sample. Instead of a single reflection from a point sample, the electric field is now a sum of all reflections with respect to depth. If we now define z such that $z = 0$ at the surface of the sample, we can update Eq. 1.2 to express the backscattered light from the continuous sample as,

$$E_s(t, \omega) \approx \int_0^\infty \frac{1}{2} \tilde{R}(z) E_0(\omega) e^{ik(\omega)(2z+2Ll_1+l_2)-i\omega t} dz \quad (1.9)$$

However, it should be noted that this is a simplified expression which firstly assumes singly scattered light, and secondly, assumes that the source electric field does not diminish with depth due to the small reflectivity of the sample. Nevertheless, we can perform the same procedure as with the point sample to obtain the intensity at the spectrometer, thus updating Eq. 1.7 to obtain,

$$I(k) = \frac{1}{8} \epsilon_0 c E_0^2(k) (1 + |\int_0^\infty \tilde{R}(z) e^{i2kz} dz|^2 + \int_0^\infty \tilde{R}(z) e^{i2kz} dz + \int_0^\infty \tilde{R}^*(z) e^{-i2kz} dz) \quad (1.10)$$

To simplify the calculation of $\tilde{R}(z)$, both sides of the equation can be divided by the source spectrum, before performing the inverse Fourier transform, like so,

$$\frac{I(k)}{\frac{1}{8} \epsilon_0 c E_0^2(k)} - 1 = |\int_0^\infty \tilde{R}(z) e^{i2kz} dz|^2 + \int_0^\infty \tilde{R}(z) e^{i2kz} dz + \int_0^\infty \tilde{R}^*(z) e^{-i2kz} dz \quad (1.11)$$

Assessing the inverse Fourier transform of the first term on the right-hand-side is simplified by remembering the Wiener-Khinchin theorem, which relates the Fourier transform of the absolute square of a function to its autocorrelation. The second and third terms are simplified by recalling the definition of the delta function, which indicates that,

$$\delta(x - \alpha) = \frac{1}{2\pi} \int_{-\infty}^{\infty} e^{ip(x-\alpha)} dp \quad (1.12)$$

Ultimately, the result of the inverse Fourier transform containing the depth-resolved information about the sample reflectivity $\tilde{R}(z)$ is,

$$\frac{I(k)}{\frac{1}{8}\epsilon_0 c E_0^2(k)} - 1 = \sqrt{\frac{\pi}{2}} \tilde{g}\left(\frac{\zeta}{2}\right) + \tilde{R}\left(\frac{\zeta}{2}\right) \text{ for } \zeta > 0 \quad (1.13)$$

where the first term is simply autocorrelation noise.

The 1D sample reflectivity over (x,z) is termed an A-scan. Repeated scanning over the same (x,z) position is termed M-mode scanning, whereas the 2D information can be obtained by scanning along one dimension. This 2D image is termed a B-scan. Finally, a 3D image can be obtained by merging several B-scans obtained over different y-positions. Figure 1.2 gives an overview of OCT scanning.

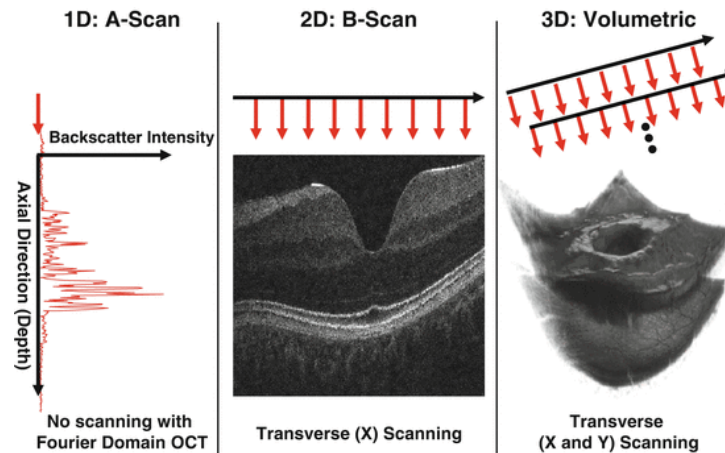


Figure 1.2. OCT scanning and scanner coordinate system. *Left:* 1D acquisition (A-Scan). A single depth profile is acquired which measures backscattered intensity vs. axial dimension (depth). *Middle:* 2D imaging (B-Scan). The OCT beam is scanned in a transverse direction while A-Scans (*red arrows*) are acquired. *Right:* 3D acquisition. Multiple B-Scans are acquired such that A-scans are sampled on a 2D grid in the transverse plane. Reproduced from [16].

1.4. OCT Angiography

OCT is widely used to visualize the vasculature. One key advantage of OCT for imaging of the vasculature over other common imaging modalities such as multi-photon fluorescent microscopy is that no extrinsic contrast agent is required. In OCT, vessel contrast is obtained by the dynamic passage of red blood cells (RBCs) in vessels as opposed to the surrounding tissue which remains relatively static. Thus, in essence, angiograms can be obtained by assessing which areas have changed when comparing two images of the same region separated by a small time delay.

The time interval between images has to be carefully chosen to balance the trade-off between sensitivity and noise [17,18]. Longer time delays will enable the visualization of vessels in which blood flows slowly, resulting in slow signal decorrelation, but may be more susceptible to increased background noise. The design of the scanning protocol must take into account the expected flow velocities to adequately satisfy the time delay constraints. Commonly, this is

achieved by repeating B-scans at the same y-position, which tends to yield an optimal time delay of about 5-15 ms.

Following reconstruction of the OCT signal as described in Chapter 1.3, we obtain a complex signal which can be expressed as the following for an A-scan,

$$R(x, z, t) = |R(x, z, t)| \exp(i\phi(x, z, t)) \quad (1.14)$$

where $|S(x, z, t)|$ is the magnitude of the signal, from which the intensity can be calculated as the magnitude squared, $I(x, z, t) = |R(x, z, t)|^2$, and ϕ is the complex phase of the signal. The amplitude, intensity, phase and/or complex signal can be manipulated to obtain angiograms.

One of the earliest and simplest methods of visualizing the vasculature, named inter-frame speckle variance [19], is based on the variance of the intensity signal, which does not require any phase information as,

$$SV(x, z) = \frac{1}{N} \sum_{n=1}^N |I(x, z, n) - \bar{I}(x, z)|^2 \quad (1.15)$$

where n is the number of the OCT scan over (x, z) , N is the total number of acquisitions over (x, z) , and $\bar{I}(x, z)$ is the time-averaged intensity. This method typically does not require extensive motion correction and is not plagued by noise arising from the phase of OCT signals. On the other hand, it generally requires dense scanning which can be time-consuming and resource-demanding, and furthermore may not be as sensitive to blood flow as other methods introduced subsequently.

Alternatively, only the phase of the OCT signal can be used to generate angiograms [20]. This method was only adopted after intensity-based algorithms because of the problem posed by motion artifacts. Makita et al. introduced important innovations in motion correction which made it possible to obtain high quality images: 1) compensation of bulk shift by correcting the axial

shift between adjacent A-scans and 2) compensating the motion between neighboring B-scans [20]. Similar to Eq. 1.15, the phase-based angiogram over position (x, z) can be obtained as:

$$PV(x, z) = \frac{1}{N} \sum_{n=1}^N |(\Delta\phi(x, z, n) - \bar{\Delta\phi}(x, z))|^2 \quad (1.16)$$

where the phase difference at each (x, z) is given by:

$$\Delta\phi(x, z, n) = \phi(x, z, n + t) - \phi(x, z, n) \quad (1.17)$$

where t is the time delay between successive B-scans.

Lastly, the full complex signal consisting of both the amplitude and phase can be utilized to construct the angiogram [21]. This improves sensitivity of flow detection, especially when changes in OCT signal predominantly occur in the phase, while also being less vulnerable to bulk motion due to the innovations in motion correction. This algorithm can be implemented as described for intensity and phase-based algorithms or based on the inter-frame difference like,

$$S(x, z, n) = |R(x, z, n + t) - R(x, z, n)| \quad (1.18)$$

This technique is often referred to as optical microangiography (OMAG) and has been applied to a number of different applications including the visualization of microvasculature in the human retina [22], skin tissue beds [23], and mice cortices [14].

For studying the vasculature of the brain, OMAG has been applied to stroke [24], capillary stalling [25], anesthesia [26], neurovascular coupling [27], among other applications. Typically, OMAG is analyzed in a 2D manner by examining the maximum intensity projection (MIP) with respect to depth (z axis). Figure 1.3 shows the diameter measurement of large pial vessels for the comparison of two anesthetic agents (isoflurane and ketamine/xylazine) on vessel diameter [26]. Figure 1.4 performs a similar type of analysis but with application to neurovascular coupling and sensory-induced changes in blood volume [27]. Figure 1.5 shows the manual detection of stalling capillary segments by repeatedly acquiring angiograms [25]. When flow is

interrupted, the vessel seems to disappear due to the mechanism of contrast which relies on the movement of blood.

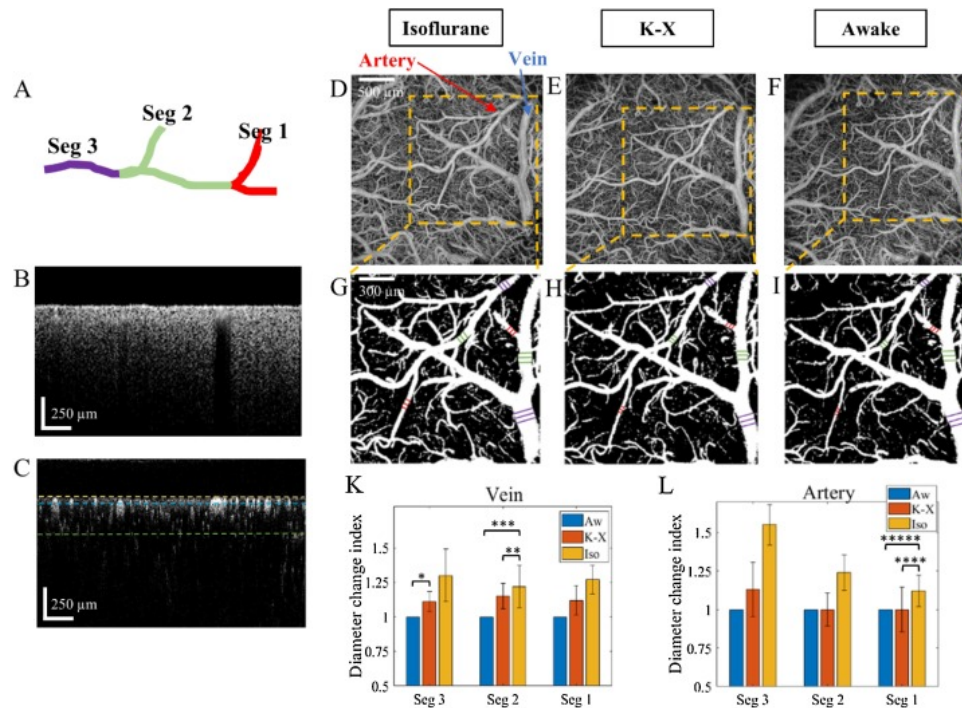


Figure 1.3. Pial vessel diameter under D) isoflurane, E) ketamine/xylazine and F) awake conditions. Reproduced from [26].

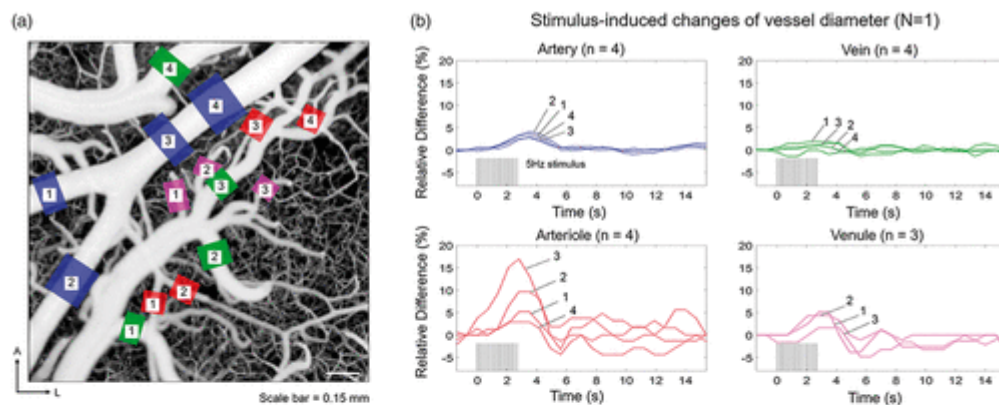


Figure 1.4. Pial vessel diameter changes in response to sensory stimulus. Adapted from [27].

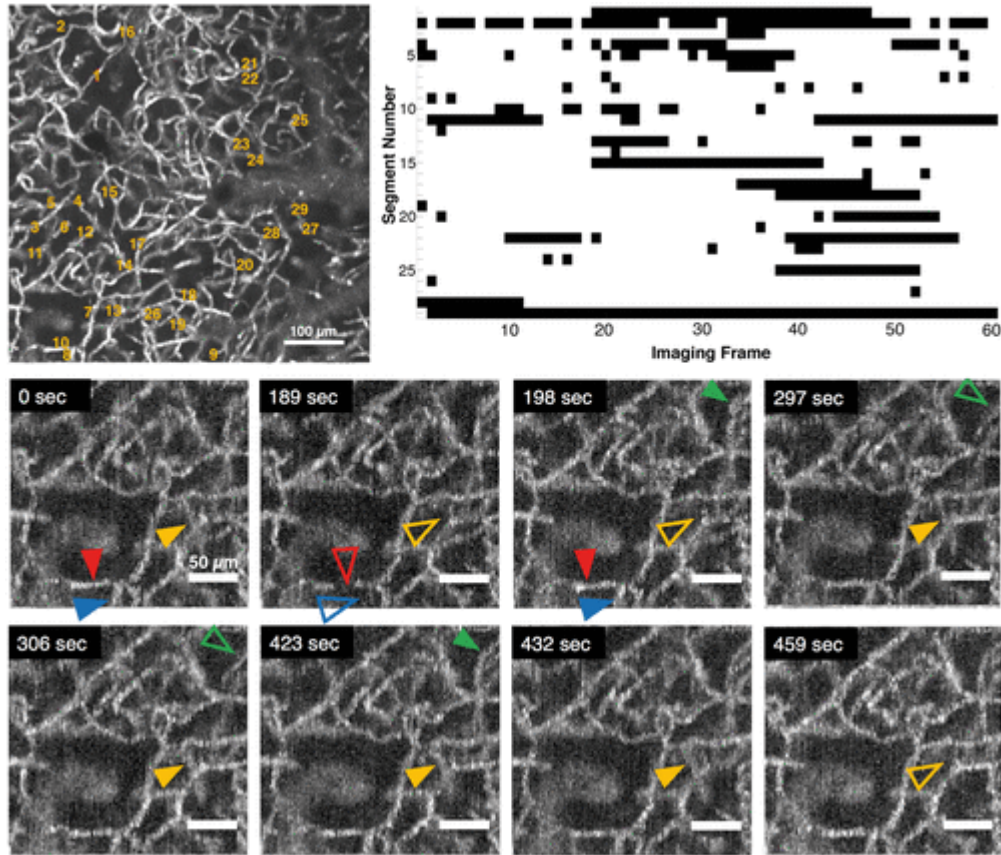


Figure 1.5. Identification of capillary segments which are stalled, i.e. blood flow is temporarily interrupted. Reproduced from [25].

1.5. Doppler OCT

OCT can also be implemented to quantify blood flow in large vessels which are parallel to the imaging beam. This is accomplished by leveraging the Doppler shift which describes the linear changes in phase which occur with axial translation of the scatterers. This can similarly be understood as the rotation of the OCT signal in the complex plane, or an offset of the power spectral density. In theory, the velocity of blood flow can be simply determined by calculating this rate of rotation, or equivalently, the slope of the phase with respect to time. Most commonly, this is performed using the Kasai estimator which can be calculated using the phase change in the autocorrelation function with a time lag equal to the time delay between A-scans [28]. More specifically, the Kasai estimator can be calculated as,

$$\hat{\Omega} = \frac{1}{2\pi\Delta t} \angle(\sum_{n=1}^{N-1} \tilde{R}_{n+1} \tilde{R}_n^*) \quad (1.19)$$

where $\angle$ indicates the complex angle. This is related to the axial velocity of blood flow by

$$v_z = \frac{\lambda_0}{2n} \hat{\Omega} \quad (1.20)$$

where n is the index of refraction of the medium and λ_0 is the central wavelength of the light source. However, this only yields the axial component of velocity of blood flow in a vessel as the angle of the vessel with respect to the imaging beam must be known to calculate the total velocity. However, the quantitative flow within a vessel can be obtained by summation of the axial velocity across the en-face cross-section of the vessel as shown in Figure 1.6.

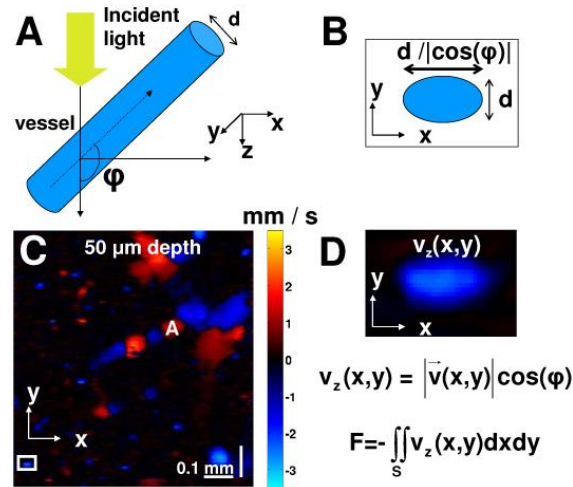


Figure 1.6. Blood flow estimation within a vessel without explicit information about the vessel orientation. Instead, only the vessel and cross-sectional area over (x,y) are needed. Reproduced from [15].

While Doppler OCT has shown to be a useful tool in a number of applications, some limitations exist: 1) axial velocity must be sufficiently large to produce a signal, 2) Doppler OCT may have limited usefulness in small vessels, particularly in capillaries where red blood cells flow in single file, 3) Doppler OCT may not reflect the true “flow profile” due to the Fåhræus effect which describes a cell-free plasma layer along the vessel walls [29]. Nevertheless, Doppler has

successfully been applied to understand cortical vascular phenomena, such as the example of ischaemic stroke [24] shown in Figure 1.7.

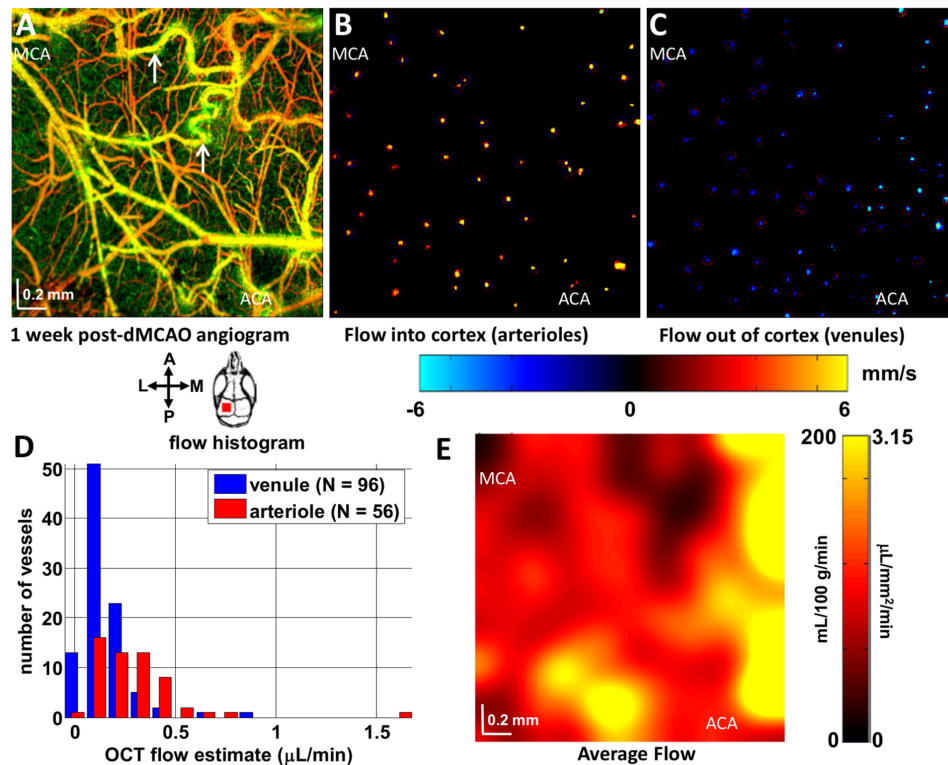


Figure 1.7. Two-dimensional maps showing the axial projection of velocity in vessels either supplying (B) or draining (C) the cortex. Reproduced from [24].

1.6. RBC tracking

While Doppler OCT cannot be used as described previously to measure blood flow in capillaries, some effort has been made to adapt Doppler OCT for this purpose, as well as develop new OCT methods. To adapt Doppler OCT for capillary velocimetry, Tang et al. aimed to address the problem of discontinuity in the flow of individual RBCs in capillaries [30]. They identified time periods at which a RBC passage was likely occurring, and calculated the axial

velocity only during these periods. Figure 1.8 shows the result of their strategy over a range of acquisition times.

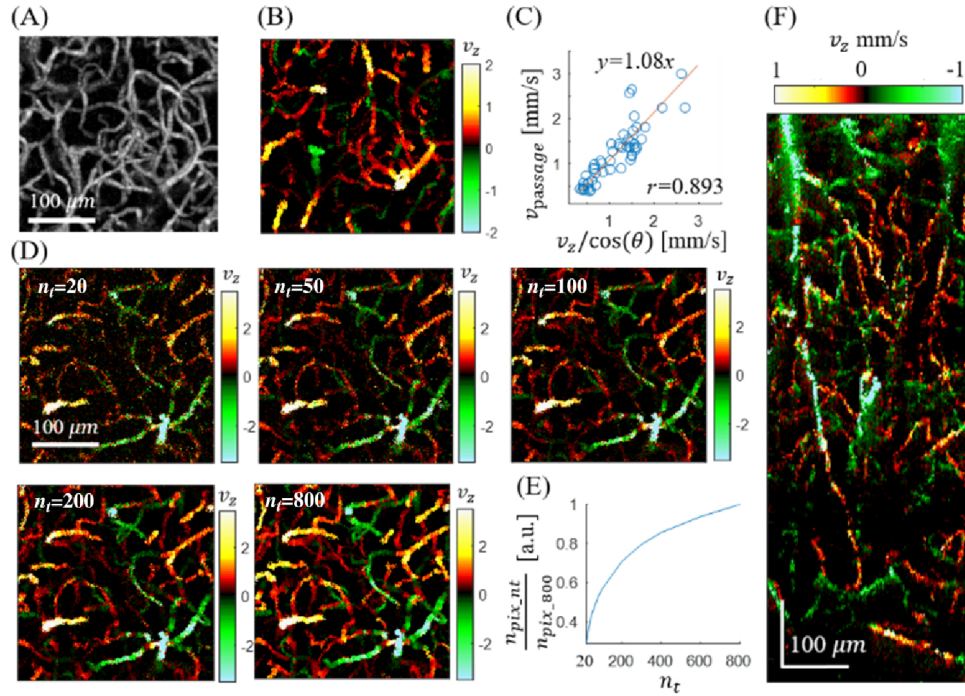


Figure 1.8. Doppler OCT adapted for capillary velocimetry. Reproduced from [30].

However, this method still suffers from the similar limitations as conventional Doppler OCT, especially the problem of only obtaining velocity in the axial dimension. Furthermore, while it may be useful to measure RBC velocity, RBC flux has been shown to be more closely correlated to tissue oxygenation [31]. For this reason, methods for the determination of RBC flux may have more applicability. Such methods are based on the observation that a transient change in phase/ intensity occurs during the passage of RBCs through a voxel [32,33]. For low-to-medium flux capillaries exhibiting sufficient signal-to-noise ratio, RBC flux can accurately be determined by simply counting the number of peaks, as validated by simultaneously-acquired OCT and two-photon microscopy measurements [34]. Figure 1.9 shows proof-of-concept for determining RBC flux in a network of capillaries based on peak-counting of transient intensity peaks [32].

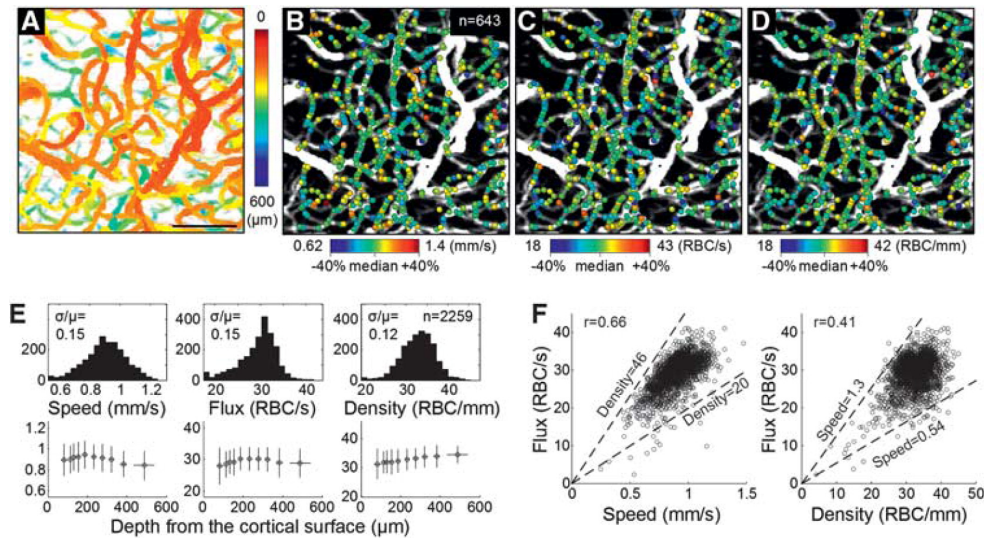


Figure 1.9. RBC flux within a network of capillaries shown in C). Reproduced from [32].

However, it has been shown that peak-counting tends to underestimate RBC flux in high-flux capillaries, and is furthermore dependent on user parametrization (such as a threshold choice) to identify “true” passages of RBCs. Chapters 4 and 5 of this thesis provides further detail on these problems, as well as the proposed solutions.

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2. Determination of confocal profile and curved focal plane for OCT mapping of the attenuation coefficient

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2.1. Abstract

The attenuation coefficient has proven to be a useful tool in numerous biological applications, but accurate calculation is dependent on the characterization of the confocal effect. This study presents a method to precisely determine the confocal effect and its focal plane within a sample by examining the ratio of two optical coherence tomography (OCT) images. The method can be employed to produce a single-value estimate, or a 2D map of the focal plane accounting for the curvature or tilt within the sample. Furthermore, this method is applicable to data obtained with both high numerical aperture (NA) and low-NA lenses, thereby furthering the applicability of the attenuation coefficient to high-NA OCT data. We test and validate this method using standard samples of Intralipid 20% and 5%, improving the accuracy to 99% from 65% compared to the traditional method and preliminarily show applicability to real biological data of glioblastoma acquired *in vivo* in a murine model.

2.2. Introduction

Optical coherence tomography (OCT) is an imaging technology that has gained popularity especially in recent years due to its capability to produce high-resolution (on the order of micrometers), label-free, three-dimensional images *in vivo* [1]. OCT is an interferometric technique based on the back-scattering of near-infrared light, allowing various structures within an image to be visualized by differences in optical properties [2]. OCT thus easily lends itself as an effective tool to probe optical properties of samples, including the attenuation coefficient: a measure of the degree of attenuation of light within a sample. Several studies have demonstrated the usefulness of this measure in a host of various medical contexts, namely the quantification of ovarian collagen content [3], detection of cerebral edema [4], diagnosis of cancer [5, 6], bladder biopsies [7], quantification of atherosclerotic plaque [8, 9], glucose monitoring [10–12], etc. There is also a growing interest in tools for functional mapping during

surgery such as the use of OCT intraoperatively, for example in the delineation of cancerous and healthy tissue in the brain during surgery [13, 14]. The accuracy of resection during brain surgery is especially important in eloquent areas, such as in the speech and motor areas, where it is imperative to minimize surgery-inflicted deficits due to the removal of non-cancerous [15]. One recent study proposed to detect cancer infiltration by thresholding the attenuation coefficient, demonstrating the potential importance of high-resolution mapping of the absolute value of the attenuation coefficient [13]. In such applications, it becomes especially critical that the attenuation coefficient is calculated accurately and robustly.

Ordinarily, the attenuation coefficient is calculated by modeling the OCT signal using Beer's law, which is a single-scattering model that describes signal attenuation through exponential decay [16]. Although another model has been introduced to additionally encompass multiple scattering in highly scattering samples, generally the contribution of multiple scattering to the OCT signal of biological tissue is fairly small [17, 18]. However, an important component that needs to be considered when calculating the attenuation coefficient from OCT data is the confocal effect, which arises from focusing of Gaussian beams for transverse resolution. The modulation of OCT signal by confocal effect has been characterized in literature and depends on two factors: the position of focus within a sample, and the apparent Rayleigh range [19]. The position of focus is often simply determined by moving the sample by a known distance, assuming that the focal plane is flat; however, the robustness and accuracy of such methods are questionable as detailed below, especially when a 2D map of the attenuation coefficient is desired. Such mapping with high transverse resolution can lead to a particularly severe confocal effect, which has limited the use of the attenuation coefficient when using high numerical aperture (NA) lenses that have small Rayleigh ranges, thus making it critical to accurately specify the position of the focal plane [20]. Addressing this issue would extend applicability of the attenuation

coefficient in clinical usage, where high magnification lenses are required to examine tissue microstructure.

Commonly, the focal plane position is found manually during imaging by moving the surface of the sample through the maximum field of brightness [21]. This method relies on assumptions of the behavior of the beam in the sample; when moving the lens (or the sample), the focal plane is shifted by an equal distance within the sample. This assumption, however, is not accurate when the refractive index of the sample is different from that of the air (the case in most biological samples). Regarding the confocal profile, existing studies have opted to ignore it completely [4, 11, 12] or otherwise employed methods that avoid explicit determination of the profile, like focusing the focal plane on the surface of the sample [3, 9]. Another method involves normalizing the OCT signal by a signal of a reference sample with known attenuation coefficient, in theory effectively canceling the dependence on the confocal effect [8, 22–24]. However, this requires an additional reference sample and is only valid if the position of the focal plane is identical between the sample of interest and the reference sample, which is especially critical for high-NA lenses but not always possible. A recent study suggested to measure both the position of the focal plane and the confocal profile through curve-fitting with a simple model, but it still uses the above inaccurate assumption of equal shift and furthermore can only produce a single-value estimate, as such data without averaging is too noisy to allow for the focal plane to vary with (x,y) positions [8, 20]. The single-value determination after averaging all A-scans of a volume into a single A-scan, based on the assumption of flat focal plane, can be problematic as the shape of the confocal profile becomes distorted due to the focal plane often lying at an angle within the sample and/or having a curvature when high-NA lenses are used. Furthermore, averaging is not appropriate if the sample is heterogeneous or consists of various structures or high-resolution 2D mapping is desired.

These issues have led us to develop a method to produce high-resolution 2D maps of the attenuation coefficient by accurately determining the positions of the focal plane within a sample while considering the confocal effect and allowing for curved focal planes, thus being applicable to OCT data taken with both low- and high-NA lenses. This method allows for either a single-valued estimate of the focal plane position or a 2D map of the focus positions to account for the curvature and tilt within the sample. We compare these methods to the standard method [20, 24] by calculating attenuation coefficient maps of standard samples of Intralipid 20% and 5% using both high-NA and low-NA lenses. Lastly, we apply the newly-developed method to produce an attenuation coefficient map of a murine xenograft glioblastoma, demonstrating applicability to real biological data.

2.3. Methods

2.1.1. OCT system

All OCT measurements were collected with a commercial SD-OCT system (Thorlabs, Newton, NJ, USA). The system uses a large-bandwidth near-infrared light source with center wavelength of 1310 nm, and wavelength bandwidth of 170 nm, which leads to a high axial resolution of 3.5 μm . The system uses a high-speed 2048-pixel line-scan camera to achieve 147,000 A-scan/s with a relatively large imaging depth (2.5 mm maximum). Two objective lenses were tested: low-NA lens (LSM03, Thorlabs) with the focal length of 36 mm, Rayleigh range (zR) of 135 μm , and the transverse resolution of 25 μm with the 1310-nm wavelength (both in air); and high-NA lens (10X Mitutoyo Plan Apo NIR Infinity Corrected Objective, Mitutoyo) with the focal length of 20 mm, Rayleigh range of 40 μm , and the resolution of 5 μm . The experimentally-determined values of the NA are 0.053 and 0.27 for the low-NA and high-NA lens, respectively.

2.1.2. Scanning and reconstruction of OCT data

When using the low-NA lens, we scanned samples with the lateral sampling interval of 3 μm , while a lateral sampling interval of 1.5 μm was used for the high-NA lens. This volumetric scan was repeated 2 times for later volume averaging to reduce speckle noise. The lens was moved a distance of about 0.1mm (such that the focal plane was still within the sample) and the imaging process repeated. Acquired fringe data were reconstructed to 3D images of the OCT intensity following the widely-used method that involves numerical dispersion compensation, k-space interpolation, and inverse Fourier transformation [25]. The reconstructed 3D images underwent a preprocessing to reduce noise: median filtering (kernel: 3x3x3 voxels), volume averaging, and then Gaussian filtering (kernel: normalized Gaussian with 1x1x1 voxel widths in σ and 5x5x5 voxels in window size).

2.1.3. Standard samples

To assess the performance of the proposed and existing methods, we test the methods on solutions of Intralipid with concentrations of 20% and 5% (v/v). Intralipid is a fat emulsion and standard sample that has been used extensively within literature because it has attenuation coefficients in the same range as biological tissue [26, 27]. The attenuation coefficients are well described in literature [28] and are referred to in this paper as μ_t^{ref} , such that $\mu_t^{ref} = 4.7 \text{ mm}^{-1}$ for Intralipid 20% and $\mu_t^{ref} = 2.3 \text{ mm}^{-1}$ for Intralipid 5%. Using each lens, for each concentration of Intralipid (20% and 5%), two datasets were taken such that the datasets were related by a vertical translation of the lens of about 0.1 mm. The vertical translation was controlled by a motorized translation stage (Thorlabs, K-Cube DC Servo Motor Controller) which was integrated with the OCT system to position the OCT probe.

2.1.4. Xenograft glioblastoma animal models

The mouse model for glioblastoma involved the growth of human-derived tumor in immunosuppressed mice for its superior predictability in location and expected growth over genetic models as well as the selection of cells that constitute the tumor in question. These xenograft models grant said consistency in tumor development and location without greatly sacrificing fidelity to the disease as these models retain invasive growth patterns mimicking human glioblastoma. Additionally, these injections require the use of immunocompromised "nude" mice, without which the tumor would never develop due to the body's immune system recognizing and attacking the foreign cells. As such, this absence leads to the loss of an analogous immune response. However, the replicability and certainty in tumor location and development from onset grants advantages that offset these costs. Sedation of 8-week old Nu/J mice was initiated with 4% isoflurane and continued with 2% isoflurane for the duration of the intracranial injection of human glioma stem cells (hGSCs) via a burr hole in the skull several weeks prior to imaging using a stereotaxic apparatus in collaboration with the Tapinos Lab. After leveling the skull, a hole was drilled with a #72 micro drill bit (Kyocera) at coordinates -2.0 mm AP and +1.5 mm ML relative to Bregma. A 75 RN Hamilton syringe was then lowered to a depth of -2.5 mm DV at a rate of .5 mm per minute, and 200,000 primary hGSCs resuspended in a total volume of 4 μ l were injected at a rate of 0.5 μ l per minute using a Stoelting Quintessential Stereotaxic Injector. To reduce backflow, the syringe rested for 5 minutes post-injection, before it was withdrawn at a rate of 0.5 mm per minute, and the cavity was immediately sealed with bonewax (Ethicon). The mice revived with additional oxygen and aged up to four weeks before terminal imaging via a cranial window.

2.1.5. Animal preparation and imaging

The animal was administered 2.5% isoflurane. An area of the skull overlying the somatosensory cortex was shaved, and surgical scrub was applied over the incision site to prevent contamination. A 2 cm midline incision was made on the scalp and the skin and both sides of the midline was retracted. A 6 mm area of the skull overlying the region of interest of the cortex was thinned with a dental burr until transparent (100 μ m thickness). The thinned skull was carefully removed over the region of interest by cutting the thinned skull with scissors, keeping the dura intact. A well was created around the region of interest using dental acrylic, filled with a 1% agarose solution in artificial CSF at room temperature, and covered with a small glass cover slip. With this window sealed by agarose and covered by the cover slip, the brain was protected from contamination and dehydration. A metal frame was glued by dental acrylic to the exposed skull, used to hold the head and prevent motion during microscopy measurements. After surgery, the animal was placed into the OCT system for in vivo imaging. The animal was kept under the isoflurane anesthesia during imaging. We used the scanning parameters as detailed above. Oxygen saturation, pulse rate, and temperature were continuously monitored with pulse oximetry and a rectal probe during the whole surgical procedure and experiment. The body temperature was maintained at 37 °C and the pulse rate remained within the normal range of 250-350 pulse/min. All animal-based experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Brown University and Rhode Island Hospital, according to the guidelines and policies of the office of laboratory animal welfare and public health service, National Institutes of Health.

2.1.6. Determination of focal plane: three approaches

Traditionally, single back-scattered light detected with OCT is modeled using Beer's law [16], which takes the form of exponential decay with the attenuation coefficient as the decay constant,

$$I(z) = h(z) \exp(-2\mu_t z) \quad (2.1)$$

where $I(z)$ is the intensity of light at depth from the surface of a sample, μ_t is the attenuation coefficient, and $h(z)$ is a function known as the confocal profile which describes the effect of confocal gating on the OCT signal [19]. For Gaussian beams, the confocal effect is described as,

$$h(z) = \left(\left(\frac{z - z_f}{\alpha n z_R} \right)^2 + 1 \right)^{-1} \quad (2.2)$$

where z_f is the position of the focal plane, z_R is the Rayleigh range, and n is the refractive index. The factor of α is incorporated to account for the effects of scattering on the Rayleigh range within the sample, and its value $\alpha = 2$ has been derived and widely used under the assumption that the interaction of the scatterers with the incident beam forms a secondary beam with new beam waist and Rayleigh range [19]. Although this theoretically estimated value can slightly differ from empirically reported values depending on various factors like the NA and incident angle [16], we used the theoretical value as it is a reasonable estimate for single-scatterers and positions of focus close to the surface of the sample [16, 20]. For analyzing data of the standard samples (Intralipid), $n = 1.35$ was used. To determine z_f , the proposed method involves scanning two images of the sample of interest, I_1 and I_2 , such that I_1 is related to I_2 by a small vertical shift (on the order of 0.1 mm). Once the surface of the sample is identified from each image, the images are coregistered for subsequent analysis, often by vertically shifting both and such that the surfaces in the two images are aligned. Dividing the image I_1 by the I_2 image then yields I_d which is simply a function of the two confocal effects,

$$I_d \propto \frac{I_1(z)}{I_2(z)} \propto \frac{((z-z_f - \beta \Delta z)/z_r) + 1}{((z-z_f)/z_r)^2 + 1} \quad (2.3)$$

where z_r is the apparent Rayleigh range ($z_r = \alpha n z_R$), Δz is the vertical shift, and the coefficient β accounts for the possibility that the shift of the focal plane differs from Δz . Most of the previous methods, either comparing two B-scans or moving the focus from the sample surface by a known distance, assumed that this focus shift is identical to the sample (or lens) shift [21, 29]; however, in this paper we argue that it is not true in most biological samples with different refractive indices from the air. We find that even when the refractive index is taken into account when estimating the focal shift (even though the refractive index is another unknown in many cases), the results are not significantly different (see Section 2.5 for details), such that it is optimal to allow this parameter to be a variable. To compare our newly-developed approaches to the previous methods, we implemented the assumption of equal shift in Method 1 by adopting $\beta = 1$. Method 1 also adopts the strategy of averaging all A-scans (as commonly done) and then fitting Eq. 2.3 with $\beta = 1$ to the averaged I_d to obtain a single-value estimate of z_f . To investigate the effect of varying β , we implemented Method 2 by including β as a free coefficient in the fitting of Eq. 2.3 to the averaged I_d to obtain another single-value estimate of z_f . However, the focal plane can be curved or tilted more generally. Thus, we implemented Method 3 as an extension of Method 2, where Eq. 2.3 is fitted to each of the individual A-scans, allowing us to determine z_f at each (x,y) position as a 2D map. To reduce noise in the raw 2D map of z_f , we fitted the map to a 2D second-order polynomial z_f ($S(x, y) = \text{const.} + ax + by + cx^2 + dxy + ey^2$) and used the smooth curved plane in further analysis. When fitting Eq. 2.3 to I_d to determine z_f , the region of fitting was determined as the one with the largest decay in the laterally-averaged I_d , identically in all methods. Three methods are illustrated in Fig. 2.1 and Table 2.1. Figure 2.2 further demonstrates the procedure with examples and Fig. 2.3 shows an example of the curved plane fitting. As can be seen in the example of Fig. 2.2(a), Method 1 forcing $\beta = 1$ generally results in worse fitting (red line) than Method 2 with variable (green line).

The fitting can be performed as described by Faber et al. [16]: suppose we are interested in fitting a model f with M adjustable parameters a_j to N data points (x_i, y_i) . The estimates of the model parameters a_j is found by minimizing the quantity χ^2 given by:

$$\chi^2 = \sum_{i=1}^N (y_i - f(x_i; a_1 \dots a_M))^2 \quad (2.4)$$

For non-linear models the χ^2 -minimization is an iterative process, implemented by the LevenbergMarquardt method, using the MATLAB function `lsqcurvefit`. The goodness of fit is given by R^2 , which is a measure that ranges from 0 to 1. $R^2=1$ means that the model perfectly fits the data. Simple ray-optics calculation suggests that β should be approximately identical to the ratio of refractive indices of the sample to the air (about 1.35 in our standard samples), although we show that even when the refractive index is accounted for, the focal plane position cannot always be accurately estimated (see Discussion). Empirically, our fitting result from Method 2 leads to β values ranging from 0.8 to 5.5, suggesting a wide range of values around the refractive index of Intralipid ($n = 1.35$). With employing β as a variable, the coefficient of determination of fitting has been improved overall, from $R^2 = 0.50$ (Method 1) to 0.98 (Method 2) on average.

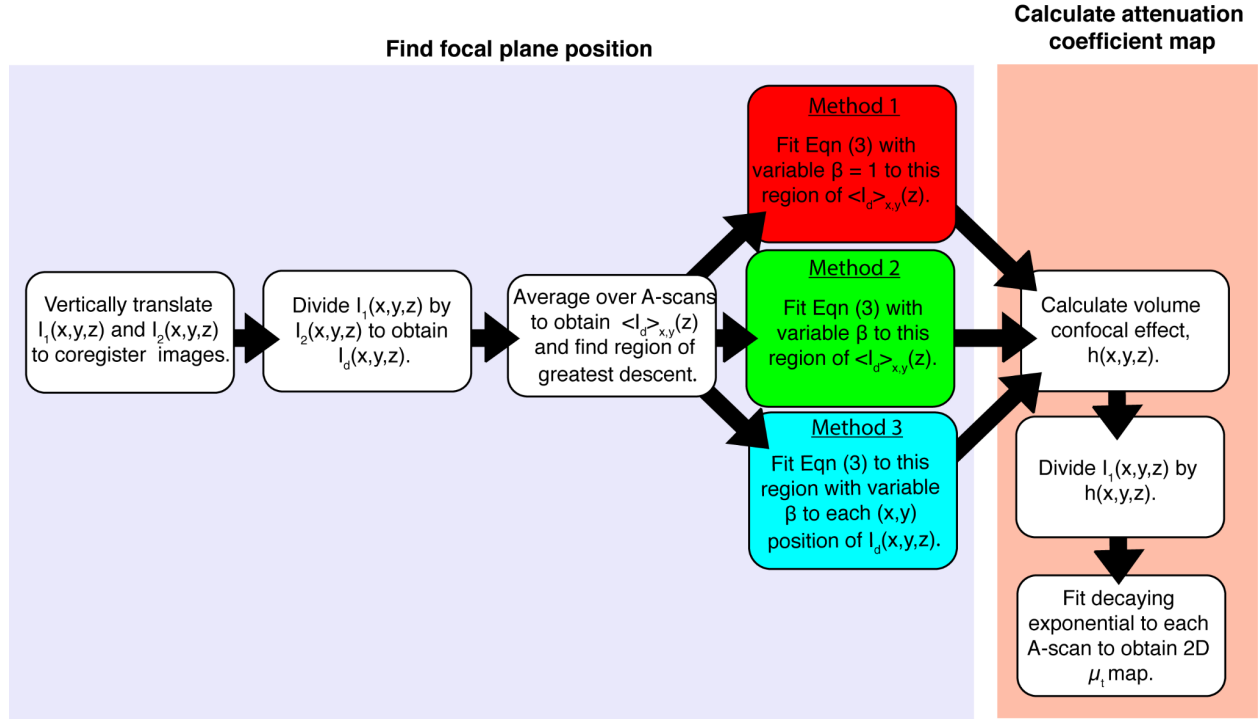


Figure 2.1. Illustration of the pipeline performed in this paper to estimate the confocal profile using the novel methods, and the application of these methods in calculating attenuation coefficient maps.

Table 2.1. Methods employed in this paper to estimate the position of the focal plane.

Method 1	Method 2	Method 3
$\beta = 1.$ Average over all A-scans to yield a single-value estimate of z_f .	β is a variable. Average over all A-scans to yield a single-value estimate of z_f .	β is a variable. Fit model to each (x, y) position of I_d to obtain 2D estimate of z_f .

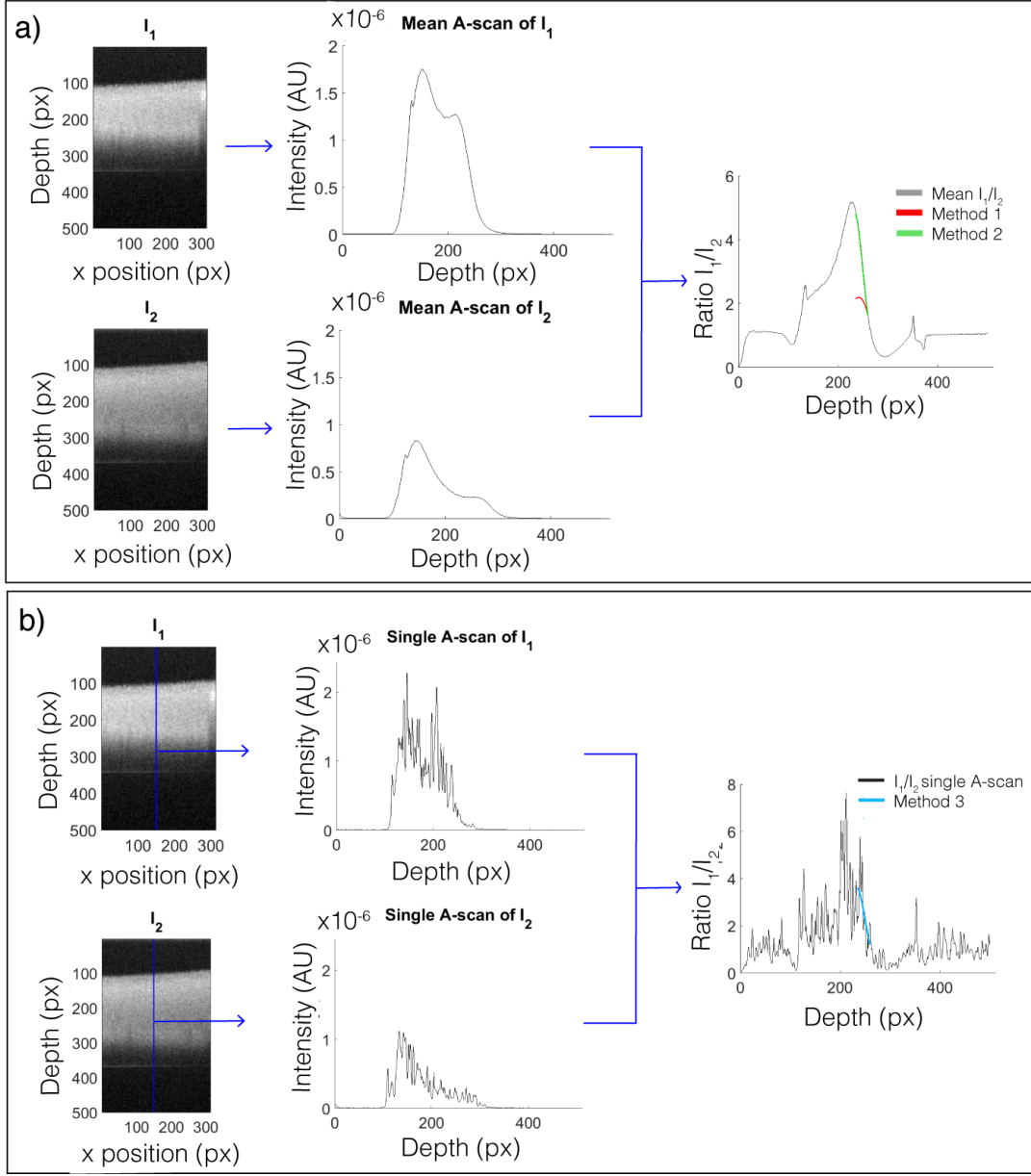


Figure 2.2. Examples of the procedures to determine z_f , where the high-NA lens and the standard sample (Intralipid 20%) were used. Left B-scan images present I_1 and I_2 (a vertical shift of the lens by about 0.1 mm) after coregistration by aligning the sample surfaces. Overlaid color lines in the right figures present Eq. 2.3 as a result of least square fittings. **(a)** Methods 1 and 2 for single-value estimates of z_f from the laterally-averaged I_d . **(b)** Method 3 for estimating as a function of (x,y) ; only one example at the specific position (the blue vertical line on the B-scan image) is presented.

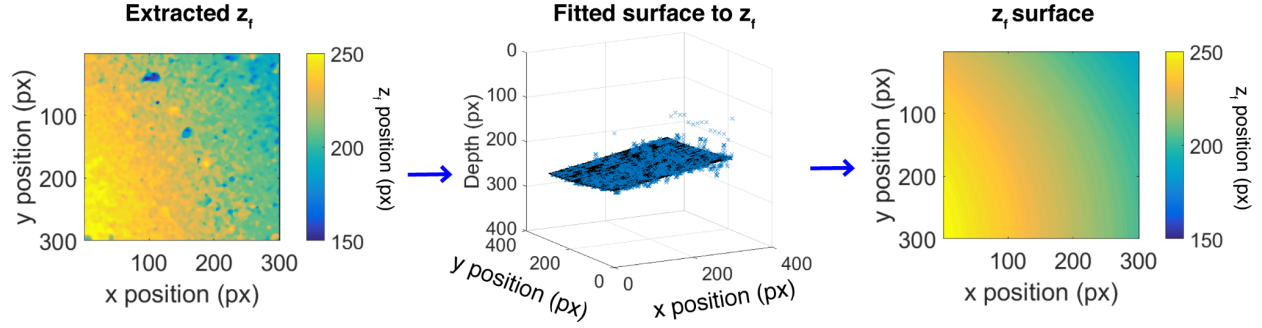


Figure 2.3. An example of the smooth-surface fitting of the individual z_f values for Method 3 using Intralipid 20% and a high-NA lens.

2.1.7. Determination of 'true' focal plane with known attenuation coefficient

To examine the focal planes determined by the three methods, we determined a theoretical focal plane by using the known attenuation coefficients of Intralipid. First, using Eq. 2.1, we extracted a theoretical confocal profile $h(z)$ by dividing the OCT image I_1 by the term $\exp(2\mu_t z)$ with the true μ_t value (Fig. 2.4). Second, theoretical $h(z)$ was smoothed to reduce noise. Then, the theoretical focus ($z_{\text{true f}}$) was determined as the position of the peak in $h(z)$, for each (x, y) position. We found that a curve-fitting approach was not possible here due to the high noise of each individual A-scan. This procedure allows us to determine the theoretical focal plane as a 2D map and use it as a reference to compare those determined by Methods 1-3.

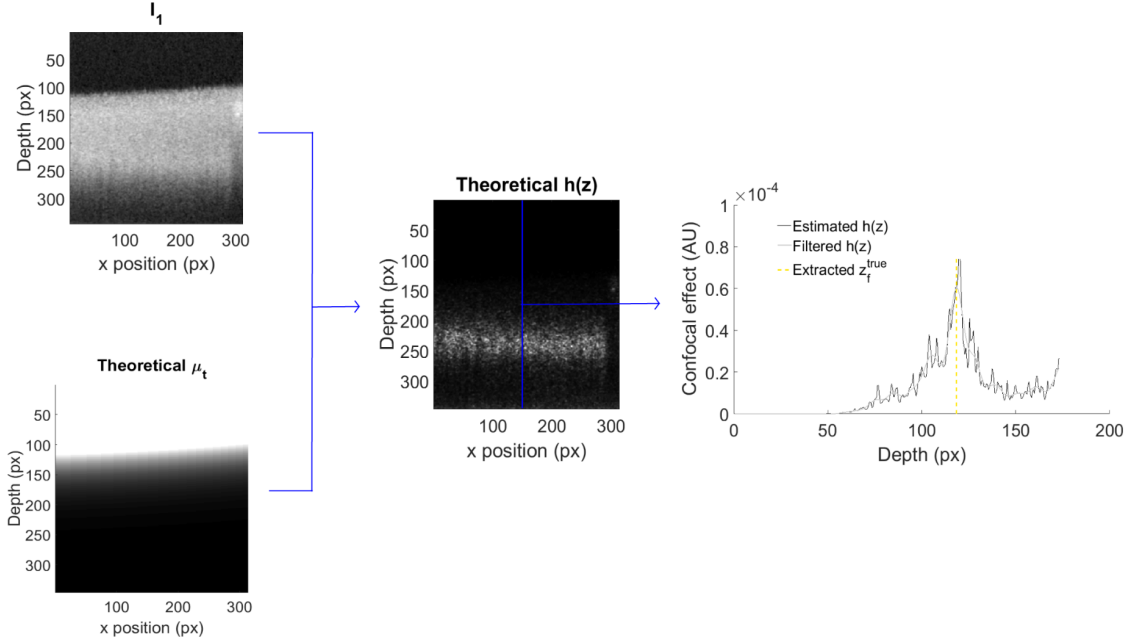


Figure 2.4. An example of the procedure to find the theoretical focal plane with the true attenuation coefficient. For this example, the same dataset as Figures 2.1 and 2.2 was used.

2.4. Results

2.4.1. Comparison of focal plane estimation

Figure 2.5 displays the focal planes determined with true attenuation coefficients (yellow), the previous Method 1 (red), our Method 2 (green), and its extension Method 3 (blue), for each combination of different scattering samples and different NAs. Method 1 based on the incorrect assumption of equal shifts not only results in wrong estimates (especially with low NA) but also cannot account for the curvature of focal planes. Although Method 2 also cannot account for the curvature, it works better in most cases. Method 3 produces the closest estimates to the theoretical planes, in all cases.

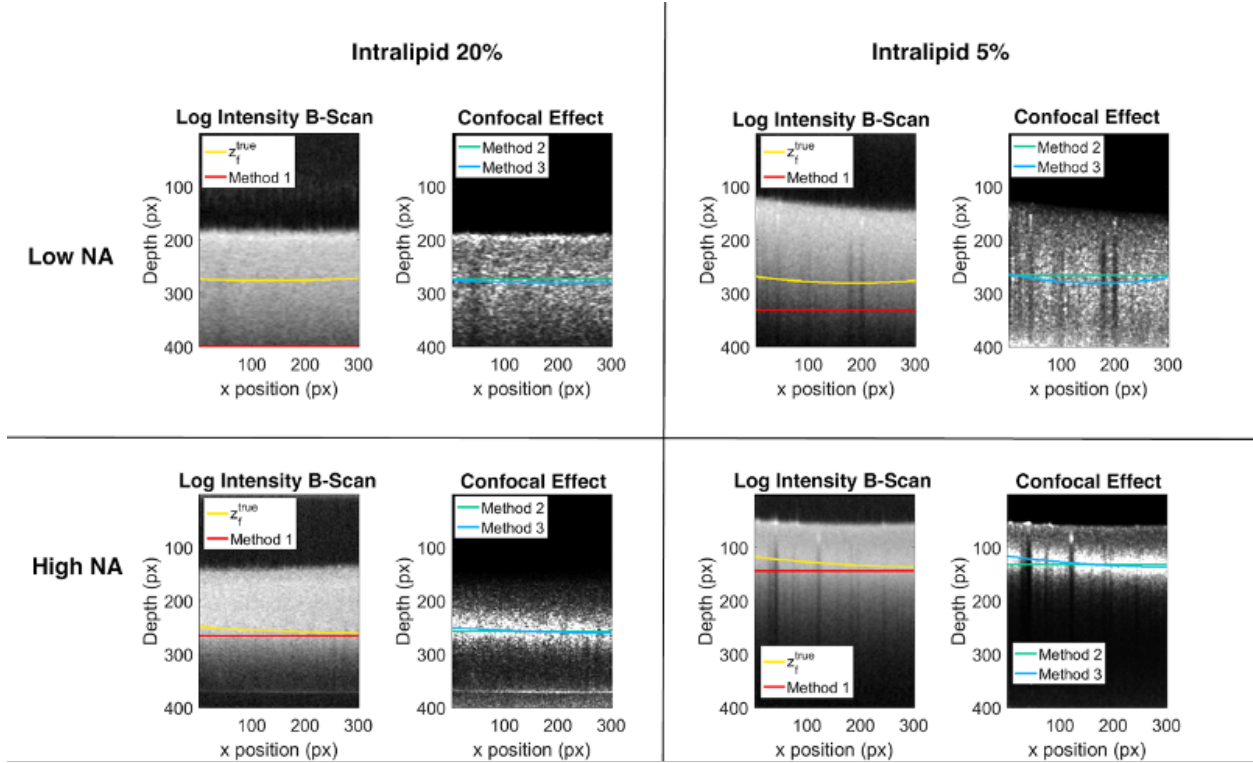


Figure 2.5. Focal planes determined with true μ_t (yellow) and Methods 1-3 (red, green, and blue, respectively). The estimations in the standard samples of Intralipid 20% and 5%, taken with both high- and low-NA lenses, are presented. For the theoretical focal planes, we used the true μ_t values as provided by literature.

2.4.2. Attenuation coefficient calculation

For each method, we used the estimated z_f value(s) to determine the confocal profile $h(z)$ from Eq. 2.2. Then, we divided the intensity map I_1 by $h(z)$ to minimize the confocal effect. Finally, the attenuation coefficient was determined by fitting the exponential component of Eq. 2.1 to each resulting A-scan, producing a 2D map of μ_t . The region of fitting for each sample was chosen by examining the log intensity of the mean A-scan. A region was chosen from the surface of the sample which appeared to be relatively linear, ensuring that the region was close to the surface of the sample to reduce the effects of multiple scattering which are amplified in depth. Figure 2.6 illustrates examples of this process for Methods 1–3. Repeating the above procedure over all (x,y) positions results in a 2D map of the attenuation coefficient. In Fig. 2.7,

we demonstrate the resulting attenuation coefficient maps produced by Methods 1, 2 and 3, as well as the distribution of μ_t . Method 1 produces not only inaccurate average μ_t values (see the histograms) but also larger variations (more heterogeneous) in attenuation coefficient from the homogeneous standard samples (see the maps). Methods 2 and 3 produce similarly accurate average μ_t values (the center of each histogram is close to the true value) with smaller variations, except for the high-NA data of Intralipid 20% sample. This result suggests that Method 3 results in more accurate μ_t measurements when high-NA lenses are used for high-resolution mapping of attenuation coefficients in highly scattering samples.

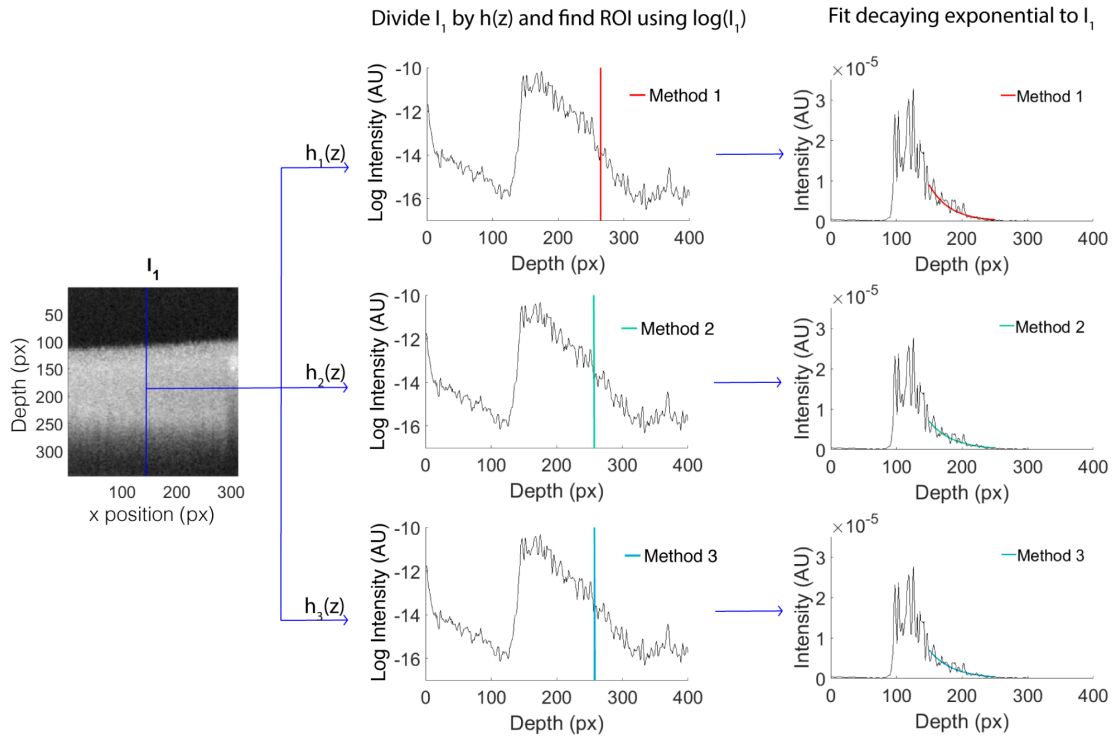


Figure 2.6. Examples of attenuation coefficient calculation from an A-scan divided by the confocal effect, for Methods 1–3. When fitting a decaying exponential to the A-scan, the region of fitting was determined by examining the region of linearity of the log intensity. The middle panel shows the estimated position of focus, and the right panel shows the fitting result.

The mean and standard deviations of the calculated attenuation coefficients are presented in Table 2. To quantify the differences in performance between the different methods, we determined the accuracy and precision of each method in each condition,

$$\text{Accuracy} \equiv 1 - \left| \frac{\langle \mu_t \rangle - \mu_t^{ref}}{\mu_t^{ref}} \right| \quad (2.5)$$

$$\text{Precision} \equiv 1 - \left| \frac{\sigma(\mu_t)}{\langle \mu_t \rangle} \right| \quad (2.6)$$

Table 2.2. Mean and standard deviations of the calculated attenuation coefficients of standard samples of Intralipid 20% and 5%.

Sample	Lens	Estimated μ_t by Method 1 (mm ⁻¹)	Estimated μ_t by Method 2 (mm ⁻¹)	Estimated μ_t by Method 3 (mm ⁻¹)
Intralipid 20% (4.7)	Low NA	6.03 ± 1.03	4.54 ± 1.47	4.70 ± 1.04
Intralipid 5 % (2.3)	Low NA	3.36 ± 0.64	2.32 ± 0.51	2.31 ± 0.48
Intralipid 20% (4.7)	High NA	6.56 ± 2.74	4.54 ± 1.47	4.65 ± 1.29
Intralipid 5% (2.3)	High NA	1.68 ± 1.07	1.90 ± 0.78	2.27 ± 0.62

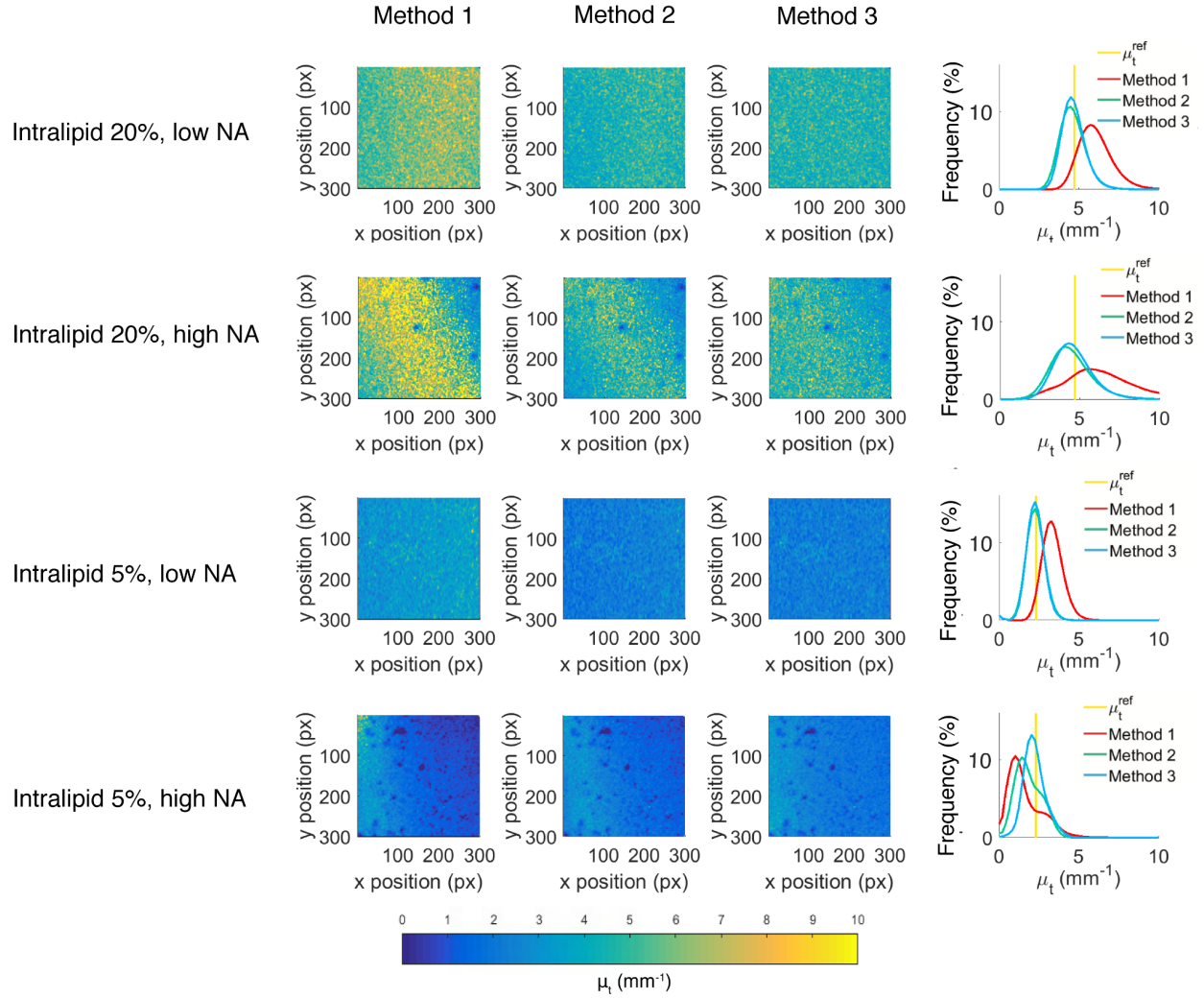


Figure 2.7. Attenuation coefficient maps obtained using Methods 1, 2 and 3. Histograms show the distribution of values for each map in comparison to the reference value, μ_t^{ref} .

2.4.3. Attenuation coefficient map of xenograft glioblastoma murine model

Having tested and validated the newly proposed methods for attenuation coefficient measurement, we apply the most accurate method, Method 3, to image the xenograft glioblastoma mouse model in vivo. Two OCT volumetric scans were performed with the low-NA lens and analyzed in the same way to the above standard-sample imaging, producing an attenuation coefficient map as presented in Figure 2.8. The difference in the attenuation coefficients between tumor and healthy tissue is very distinct in the 2D map. All the algorithms

were implemented using MATLAB, and our scripts have been uploaded to Brown Digital Repository with the DOI of 10.7301/Z0PV6HW1 for public access [30].

Table 2.3. Accuracy and precision of the three different methods tested in estimating the attenuation coefficients of standard samples of Intralipid 20% and 5% for which the attenuation coefficients are known.

		Method 1		Method 2		Method 3	
Sample	Lens	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Intralipid 20%	Low NA	71.7	82.9	96.6	67.6	100	77.9
Intralipid 5%	Low NA	53.9	81.0	99.1	77.7	99.6	79.2
Intralipid 20%	High NA	60.4	58.2	96.6	67.6	98.7	72.3
Intralipid 5%	High NA	73.0	36.3	82.6	59.0	98.7	72.7
		64.8	64.6	93.7	67.8	99.2	75.5

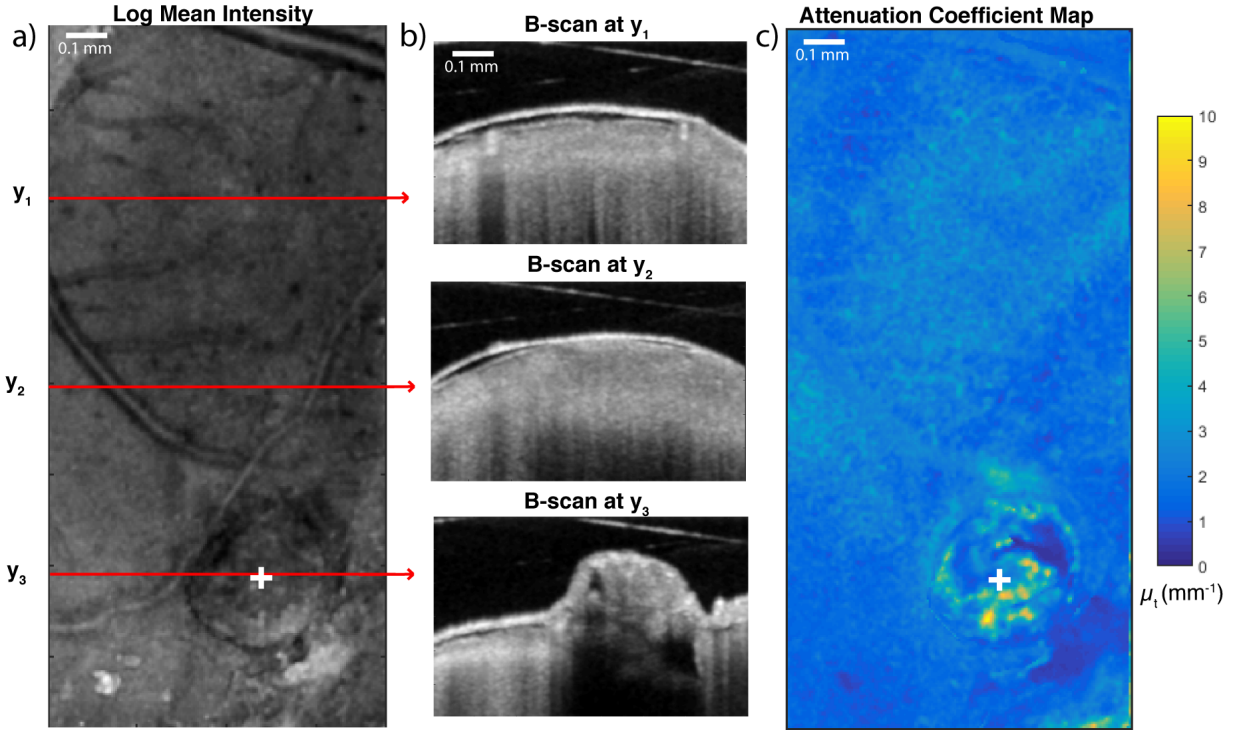


Figure 2.8. OCT intensity and attenuation coefficient maps of the rodent xenograft glioblastoma in vivo. **(a)** The *en face* OCT image of the mouse brain is presented in the log mean intensity. **(b)** Example B-scans at three y-positions (red lines in (a)) are presented in the log scale of intensity. **(c)** The attenuation coefficient map determined using Method 3. The white crossheads indicate the injection location of human glioblastoma cells. Scale bars, 0.1 mm.

2.5. Discussion

In this study, we have established and evaluated a method to robustly determine the positions of the focal plane within a sample post imaging by using two volumetric scans with a small vertical translation. By taking advantage of the fact that the attenuation coefficients are the same in the two images, the method performs a curve-fitting procedure on the ratio of the two confocal profiles to extract the focal plane position. In this course, the major improvements include that we have corrected the false assumption often made that the focus within a sample shifts in a predictable manner with the vertical translation [20, 21, 31], and we have allowed for a curved focal plane instead of the previously-used flat plane [8, 22–24]. When compared with Method 1, our method results in focal planes much closer to the theoretical one determined with

true attenuation coefficients. We further show that even when the refractive index is accounted for, it is difficult to achieve an accurate estimation of the position of focus (Table 4). It is likely that confocal effect and the behavior of light within the sample is dependent on several factors in addition to the refractive index that are not captured in theoretical models, such as the angle of incidence that the beam makes with the sample, the contribution of specular reflection, the scattering coefficient of the substance, the NA of the lens, etc. which may collectively interact to contribute to a wide spread in the β values obtained. Empirically, we have discovered that allowing beta to vary accounts for some of these effects.

Table 2.4. Accuracy and precision of Method 1 ($\beta = 1$) and Method 1' ($\beta = n$).

		Method 1		Method 1'	
Sample	Lens	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Intralipid 20%	Low NA	71.7	82.9	75.2	82.3
Intralipid 5%	Low NA	53.9	81.0	47.6	81.1
Intralipid 20%	High NA	60.4	58.2	64.5	70.9
Intralipid 5%	High NA	73.0	36.3	79.8	14.7
		64.8	64.6	66.8	62.3

Finally, the method has been shown to produce more accurate 2D maps of the attenuation coefficient in the standard samples, improving the accuracy by 34 percent points and the precision by 11 percent points. Additionally, the method provides significant improvements on data taken with high-NA lenses, in which the confocal effect contributes significantly to the modulation of the depth profiles. This may have implications for clinical use, where it is desirable to use high magnification lenses to examine tissue microstructures [24]. This study suggests that the accurate determination of the focal plane position is an important factor in precise measurement of the attenuation coefficient. We found that Method 3 which uses a 2D map of the focal plane performed significantly better than Method 1, which assumes that the focal plane shifts within the sample by a predetermined distance as defined by the lens distance. Additionally, averaging of A-scans poses a problem since it is difficult to extract a single-value estimate of the position of the focal plane from the averaged A-scans of a homogeneous sample because often the focal plane is curved or slanted within the sample, such that $h(z)$ is distorted and Eq. 2.1 is not an appropriate description of the data. If instead the sample were homogeneous and the focal plane perfectly flat within the sample, simple averaging would be theoretically possible. However, if the sample is heterogeneous (as most biological tissues are) simple averaging is once again not appropriate, even when the focal plane is flat, because the attenuation coefficient varies at different (x, y) positions, i.e. the second term in Eq. 2.1 is no longer appropriate.

By determining a 2D map of the focal plane, the attenuation coefficient maps of the Intralipid solutions are more homogeneous, as expected. We note that it appears that when lateral averaging is performed before curve-fitting to obtain a single μ_t high accuracy may be achieved as demonstrated in several papers [3, 6, 16, 28]. Despite this, the method fails if a 2D attenuation coefficient map is desired, as the results in Table 3 indicate. The results are

particularly improved in the high-NA case, in which the Rayleigh range is small thus making it critical to accurately specify the position of focus.

The presented paper, however, does not estimate the apparent Rayleigh range empirically which is known to be influenced by factors such as the scattering coefficient, the angle of incidence with the sample, and multiple scattering at deeper depths [16]. For the most accurate calculations, the dependence of the effective Rayleigh range on these factors should be characterized or otherwise determined empirically. Additionally, compared to the previous methods that measure the attenuation coefficient from single OCT volume data [3, 4, 9, 11, 16], our method requires two scans with a small vertical shift, which increases the total scanning time twice and may increase the total data processing time. However, the vertical shift can be easily implemented using a motorized translation stage, and furthermore, compared to the other method that required a reference sample and identical focal depth between two samples [8, 22], our method is much easier to implement and may result in more robust measurements.

There is a growing interest in producing and utilizing 3D maps of the attenuation coefficient [21, 32]. For example, depth-resolved attenuation coefficient maps have already been employed to investigate various tissues, including atherosclerotic [33], brain [20], and esophageal [34]. These depth-resolved methods are still vulnerable to the limitations discussed previously, namely the restriction to low-NA lenses and difficulty in precisely specifying the position of focus. Once the confocal effect is accurately described using the proposed method, any method of determining the attenuation coefficient may be applied, producing more accurate estimates of the attenuation coefficient, and also allowing for the use of high-NA lenses. The method described in this paper to determine the position of the focal plane can be applied in conjunction with more complex methods of determining the attenuation coefficient, such as 3D methods or

methods that include sensitivity roll-off which describe the reduced signal-to-noise ratio with depth. Sensitivity roll-off is incorporated into the model of intensity as,

$$I(z) = S(z)h(z) \exp(2\mu_t z) \quad (2.7)$$

where $S(z)$ is the sensitivity roll-off, which was described by Vermeer et al. as a Gaussian [32], and modeled more comprehensively for SD-OCT by Yun et al. as an explicit parameter of spectrometer and CCD parameters [35]. Once sensitivity roll-off is characterized for an OCT system, any images obtained may simply be divided by this factor since there is no dependence on the sample being imaged. Finally, this paper demonstrates an example of applying the developed method to real biological data, producing an attenuation coefficient map of glioblastoma in a murine xenograft model, showing improved contrast of cancerous and non-cancerous tissue compared to the log mean intensity. Considering that the attenuation coefficient is gaining popularity as a tool in various medical applications, like drug development in human tumor spheroids and intraoperative tumor resection [13, 36], it is becoming increasingly important that the measurements are robust and accurate. Although this paper has thoroughly evaluated the new method with standard samples and showcased its applicability with the preliminary in vivo tumor imaging, more studies are required to translate the presented method into clinical applications.

2.6. Conclusion

This paper presents a method to accurately determine the confocal effect and its focal plane, as an either flat or curved plane, for OCT measurements of the attenuation coefficient. This is achieved by examining the ratio of confocal effects of two OCT images related by a small vertical translation. This paper also shows that more accurate and precise attenuation

coefficient maps can be obtained if we avoid the assumption that the focal plane moves equally with movement of the lens, as demonstrated using standard samples of Intralipid. Lastly, this paper exemplifies how this method may be applied to real biological data of a murine glioblastoma model system.

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3. Deep Learning Toolbox for Automated Enhancement, Segmentation, and Graphing of Cortical Optical Coherence Tomography Microangiograms

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3.1. Abstract

Optical coherence tomography angiography (OCTA) is becoming increasingly popular for neuroscientific study, but it remains challenging to objectively quantify angioarchitectural properties from 3D OCTA images. This is mainly due to projection artifacts or “tails” underneath vessels caused by multiple-scattering, as well as the relatively low signal-to-noise ratio compared to fluorescence-based imaging modalities. Here, we propose a set of deep learning approaches based on convolutional neural networks (CNNs) to automated enhancement, segmentation and gap-correction of OCTA images, especially of those obtained from the rodent cortex. Additionally, we present a strategy for skeletonizing the segmented OCTA and extracting the underlying vascular graph, which enables the quantitative assessment of various

angioarchitectural properties, including individual vessel lengths and tortuosity. These tools, including the trained CNNs, are made publicly available as a user-friendly toolbox for researchers to input their OCTA images and subsequently receive the underlying vascular network graph with the associated angioarchitectural properties.

3.2. Introduction

Optical coherence tomography (OCT) is a rapid, label-free, high-resolution imaging tool that has been widely used in clinical ophthalmology and is recently becoming increasingly popular and useful for neuroscientific study. OCT angiography (OCTA) is particularly popular for the visualization of cerebral vascular networks, which is based on dynamic scattering in vessels due to moving blood cells. OCT is not only a powerful visualization tool, but can also be leveraged to quantify cerebral blood flow in arterioles and venules using Doppler OCT [1], as well as blood oxygenation levels using visible-light OCT [2,3]. These capabilities make OCT well-suited for the study of various physiological and pathological phenomena in the animal brain cortex, including ischemia [4–6], hemodynamics [7–9], capillary stalling [10, 11], and angiogenesis [12,13].

The full 3D nature of OCTA, however, is currently difficult to exploit due to projection artifacts or tails underneath vessels in the axial dimension caused by multiple-scattering [8]. In particular, these tails make it challenging to ascertain if two nearby vessels are truly connected, which is important for the graphing of the underlying vascular network and subsequent quantitative analysis. These tails have been the major drawback of OCTA for decades, limiting researchers to analyzing only 2D en-face projections and hindering the potential of OCTA for quantitative analysis like vessel connectivity, length, and tortuosity in 3D. Additionally, these tails disrupt the tubular or rod-like appearance of vessels, making many traditional techniques for vessel enhancement such as Hessian-based methods yield less than optimal results on OCTAs when compared to two-photon microscopy angiograms (TPMAs) that are free from projection artifacts.

This OCTA-specific tail effect, along with the lower signal-to-noise ratio than fluorescence-based TPMA, motivate the need for OCTA-specific image enhancement and graphing techniques.

To suppress the OCTA-specific tail artifact, Leahy et al. combined dynamically-focused OCT with a high-numerical aperture lens such that multiply-scattered photons from the tail are rejected [14]. But this method requires dense scanning in depth to obtain a volumetric image, making it difficult to utilize the Fourier-domain OCT's strongest advantage that Z-axis scanning is not required over the depth of field. The dense depth scanning with OCT not only wastes the majority of voxels but also is not applicable when one needs rapid volumetric imaging to track dynamic phenomena like neurovascular coupling. Zhang et al. introduced a layer-based subtraction approach to minimize projection artifacts in the choroid layer, but this did not translate well to cortical OCTA images [15]. Alternatively, Vakoc et al. suggested applying a step-down exponential filter beneath vessels to attenuate the signal [16]. More recently, Tang et al. introduced a technique based on autocorrelation (g1-OCTA) to greatly enhance the contrast and mitigate projection artifacts [17]. However, g1-OCTA relies on the injection of a contrast agent like Intralipid and requires 1-2 orders-of-magnitude longer acquisition time and larger data size than traditional OCTA. Therefore, we present a computational approach to efficiently attenuate projection artifacts and significantly enhance image quality of cortical OCTAs, without requiring a contrast agent or additional depth scanning.

Our approach is based on deep learning which has achieved unprecedented results in numerous biomedical and computer vision problems. Convolutional neural networks (CNNs) in particular have proven to be powerful tools in medical diagnosis through effective recognition and localization of objects from images, such as pulmonary nodules from chest CT scans [18] or tumorous regions from MRI scans [19]. CNNs have also proved to be effective at denoising and enhancing CT scans [20] and ultrasounds [21], improving clinical interpretation. In our study, to enhance cortical OCTA images, we specifically drew inspiration from the recent success of

encoder-decoders in image denoising and super-resolution [22–24]. The network architecture is described by an encoder which repeatedly down-samples the image, and a decoder which then up-samples the encoded result to the size of the original image. Since the encoder removes noise by extracting relevant features, the decoded result has fewer noise artifacts. In a sense, OCTAs are corrupted by noise and projection artifacts, so we investigated whether an encoder-decoder CNN could effectively suppress tails. To this end, we developed and trained a 3D CNN on an image pair of a standard (i.e. intrinsic-contrast) angiogram and its manually segmented label such that the trained CNN outputs an enhanced, grayscale, 3D image when applied to any microangiogram. We name this trained network EnhVess.

Along with image enhancement, image segmentation (i.e. assigning labels to each voxel) plays a key role in quantitative analysis of biomedical images. Automated segmentation is considered critical to the unbiased quantification of features and objective comparison between different experimental conditions. For example, the research community concerned with retinal OCT data has long devoted appreciable attention to the automation of segmentation, considering that manual segmentation is a laborious, time-consuming task and often vulnerable to bias. For the segmentation of vessels from angiogram images, deep learning has been shown to achieve unprecedented results on retinal OCTA images [25–27] and cortical TPMAs [28–30]. However, CNNs trained on these image types perform poorly on cortical OCTAs. Thus, we developed and trained another CNN on an image pair of an enhanced angiogram produced by EnhVess and the manually-segmented label. This second network, entitled SegVess, is architecturally simple with only a few hidden layers and one skipped layer. This simple architecture prevents overfitting, while achieving high accuracy [31]. After segmentation, we employ ConVess, a third CNN which is designed to improve connectivity of the segmentation output and therefore reduce effort in correcting gaps during post-processing. This approach is entirely computational,

requiring no additional experimental data, and only takes several seconds to process while manual segmentation takes from days to weeks.

Lastly, this paper describes our strategy for graphing the vascular networks from segmented angiograms and quantifying various angioarchitectural properties. Graphing includes the process of reducing each vessel to a single line that passes through its center, which in turn enables the representation of a vascular network as a mathematical graph. From this graph, several parameters can be measured, such as the branching order, vessel length, and vessel diameter. In consequence, this paper presents a complete pipeline of enhancement - segmentation - vessel graphing - property measurement. This pipeline is offered as a toolbox that allows researchers to input their standard OCTA and receive as an output the underlying vascular graph along with the associated angioarchitectural properties.

3.3. Methods

3.3.1. OCT system

All OCT measurements were collected with a commercial SD-OCT system (Telesto III, Thorlabs, Newton, NJ, USA). The system uses a large-bandwidth, near-infrared light source (center wavelength, 1310 nm; wavelength bandwidth, 170 nm) which leads to a high axial resolution of 3.5 μ m in air. The system uses a high-speed 2048-pixel line-scan camera to achieve 147,000 A-scan/s with a relatively large imaging depth (3.6 mm in air; up to 1mm in brain tissue). The transverse resolution is 3.5 μ m with our 10x objective (NA = 0.26).

3.3.2. Animal preparation and imaging

Male wild-type mice (n = 2, 20 weeks old, 20-25g in weight, strain C57BL/6, Jackson Laboratories) underwent craniotomy surgeries following the widely used protocol [32]. All

animal-based experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Brown University and Rhode Island Hospital. Experiments were conducted according to the guidelines and policies of the office of laboratory animal welfare and public health service, National Institutes of Health.

In detail, the animal was administered 2.5% isoflurane. An area of the skull overlying the somatosensory cortex was shaved, and surgical scrub was applied over the incision site to prevent contamination. A 2-cm midline incision was made on the scalp, and the skin on both sides of the midline was retracted. A 6-mm area of the skull overlying the cortical region of interest was thinned with a dental burr until transparent (100- μ m thickness). The thinned skull was removed with scissors while keeping the dura intact. A well was created around the opened area using dental acrylic, filled with a 1% agarose solution in artificial CSF at room temperature, and covered with a small glass cover slip. With this window sealed by agarose and covered by the cover slip, the brain was protected from contamination and dehydration. A metal frame was glued by dental acrylic to the exposed skull, used to hold the head and prevent motion during microscopy measurements. After surgery, the animal was placed under the OCT system for in vivo imaging. The animal was kept under isoflurane anesthesia during imaging. Oxygen saturation, pulse rate, and temperature were continuously monitored with a pulse oximeter and rectal probe for the entire surgical procedure and experiment. Body temperature was maintained at 37 °C and the pulse remained within the normal range of 250-350 bpm. After the acquisition of the first angiogram with intrinsic contrast, an intravenous injection of 0.3-ml Intralipid was administered through the tail vein. We placed the focus about 0.5 mm from the surface of the brain, since we are particularly interested in the enhancement and segmentation of the microvascular bed.

This study used five OCTA datasets. The first two were acquired from the same cortical region, the first being an intrinsic-contrast ("standard") OCTA while the second one was obtained using

Intralipid as a contrast agent to guide the manual annotation. The first standard OCTA was manually labeled; this OCTA and its manual annotation were used for training and testing the three networks. The Intralipid OCTA was not used in training any of the networks. The other three OCTA datasets were used for investigating the performance of the trained networks on different OCTA datasets: one with a larger field of view, one with lower resolution, and another acquired with a different OCT machine by another lab.

3.3.3. Manual angiogram annotation

We manually labeled vessels in the standard OCTA while using the Intralipid OCTA as a guide. These angiograms are of size (x,y,z) $300 \times 300 \times 350 \text{ um}^3$ or $200 \times 200 \times 230$ voxels.

After acquisition, the images were registered and overlaid, allowing us to use the high-contrast Intralipid image as a guide for manual segmentation of the standard OCTA. We successively examined each slice of these overlaid images to manually label the vasculature using a pen tablet, which took three months, as shown in Fig. 3.1. The manual annotation was performed using our custom-made user interface which allows for navigating a 3D image both horizontally and vertically by traversing cross-sectional B-scans or en-face slices. We manually annotated vessels with a few criteria: (1) a line of voxels should have higher OCT decorrelation values than its neighboring voxels, when viewed en face; (2) the voxels look highly continuous within the line; and (3) the line of voxels is well connected to one or two neighboring segmented vessels when viewed in 3D space, among other intuitive guides.

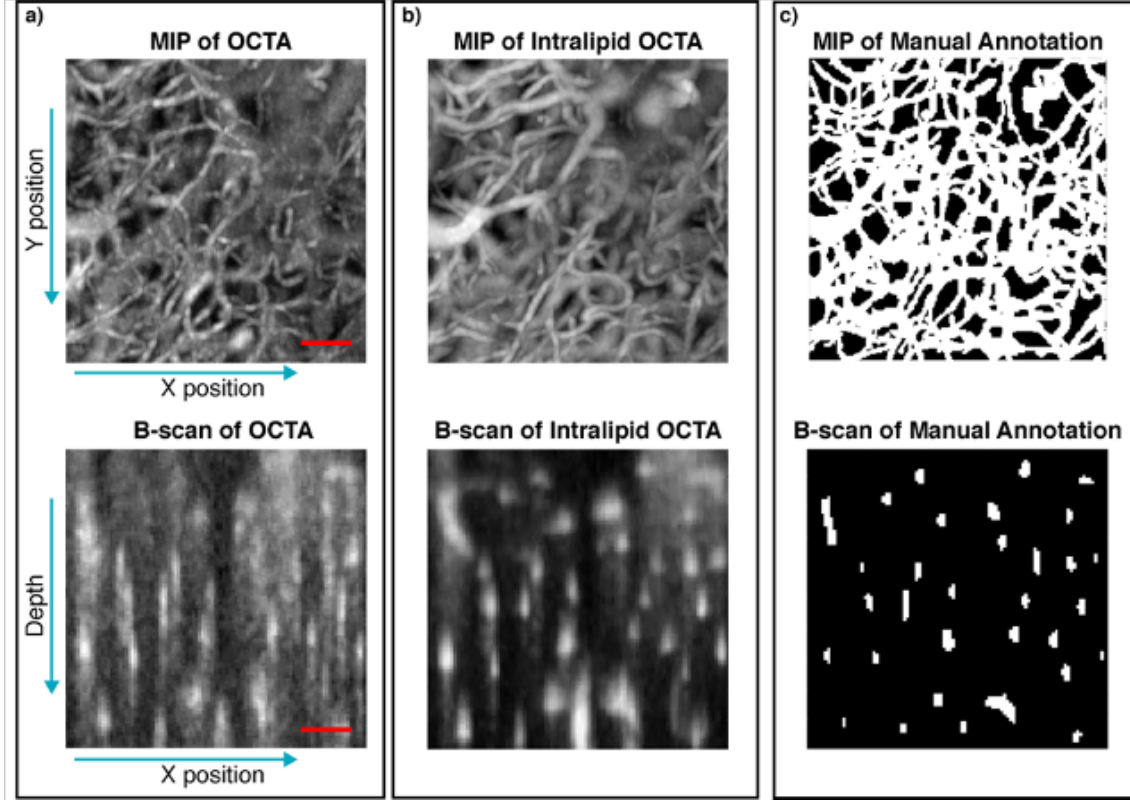


Figure 3.1. Manual annotation of OCTA with the aid of external-contrast OCTA. **(a)** Intrinsic-contrast (“standard”) OCTA. **(b)** Registered Intralipid-contrast OCTA. **(c)** Manual segmentation of the standard OCTA under the guidance of Intralipid OCTA. Scale bars, 50 μm .

3.3.4. Developing and training EnhVess: OCTA enhancement and “tail” removal

EnhVess is the encoder-decoder employed for image enhancement. This network was trained on the intrinsic-contrast angiogram with the manual annotation as the ground truth, using the mean-squared loss function. We chose this specific loss function because we sought a continuous output and this loss function is most widely used to accomplish this. The input to the encoder is a 3D patch of $32 \times 32 \times 32$ voxels of the intrinsic-contrast angiogram. This size was chosen to facilitate downsampling and subsequent upsampling by factors of 2. The encoder consists of three 3D convolutional layers followed by a max pooling layer. The output of the

encoder is then decoded by three transposed convolution layers which upsample the layer inputs and final convolutional layer to produce an output of size $32 \times 32 \times 32$. This network architecture is illustrated in Fig. 3.2. All convolutional layers employ a rectified linear unit (ReLU) as the activation function, except the final layer which utilizes a clipped ReLU function to produce a continuous output from 0 to 1. To improve performance and stability, we performed batch normalization after each layer. We trained our network using the Adam stochastic optimization for 500 epochs with a learning rate of $1e-4$.

For training EnhVess, we used 4,000 patches of $32 \times 32 \times 32$ voxels from the single OCTA volume data shown in Figs. 3.1a and 3.1c. In detail, from the intrinsic-contrast angiogram with $200 \times 200 \times 230$ voxels, we first reserved about 15% of the data (of size $200 \times 32 \times 230 [x,y,z]$) and did not use it in training either EnhVess or SegVess, allowing the networks to be tested on the unseen data. From the other part of the intrinsic-contrast angiogram ($200 \times 168 \times 230$ voxels), 500 patches of $32 \times 32 \times 32$ voxels were randomly obtained. This dataset was augmented by random rotations around the z-axis as well as horizontal flipping, which increased the size of the training data by a factor of 8 (4,000 in total).

When EnhVess was applied to a new, unseen OCTA ($512 \times 512 \times 350$ voxels) as shown in the Results, the OCTA was split into $32 \times 32 \times 32$ patches, and each patch was processed by the network.

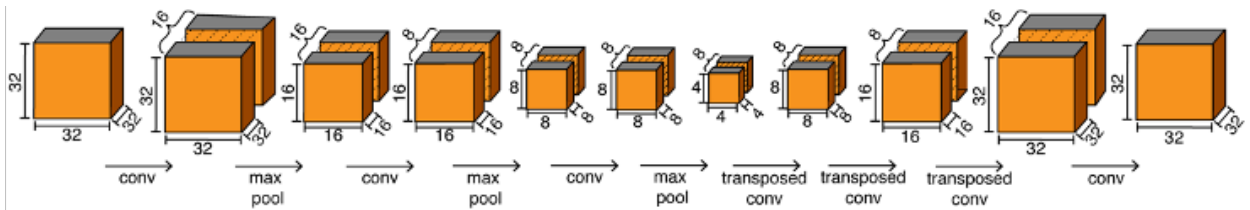


Figure 3.2. Architecture of the encoder-decoder used in EnhVess for volumetric image enhancement and “tail” removal.

3.3.5. Developing and training SegVess: OCTA segmentation

Once EnhVess was fully trained, we trained a second CNN, SegVess, for vessel segmentation using the output of EnhVess (the enhanced image) as the input along with the labeled data as the ground truth. In an attempt to use a much smaller number of learnable parameters compared to large networks such as U-Net that has about 30 million, SegVess consists of 4 hidden layers and one skip connection, as shown in Fig. 3.3. As a result, this simple architecture has about 1% of the number of learnable parameters. Like EnhVess, we employed 3D convolutions and the ReLU activation function. However, SegVess was designed to produce a binary output (vessel vs background) using the class probabilities determined by the final softmax layer. This network was trained using Adam stochastic optimization and soft Dice loss. The Dice loss has been shown to be effective for imbalanced classes, which is critical here since the vasculature only fills a portion of the total volume (typically less than 10%). To prevent overfitting and improve accuracy, we performed batch normalization after each convolutional layer. Like with EnhVess, we augmented the training data by rotations around the z-axis, as well as horizontal flipping.

To train SegVess, we used 16,000 patches of $16 \times 16 \times 12$ voxels from the enhanced OCTA image shown in Section 3.1 below. In detail, we first split the data into 3 sets for training, validation and testing. The training data was of size $200 \times 136 \times 230$ voxels, the validation data of size $200 \times 32 \times 230$ voxels, and the testing data of size $200 \times 32 \times 230$ voxels. Thus, SegVess was trained with the same ground truth as EnhVess but was tested using data that was unseen to either network during training. Before training SegVess, we determined the optimal input patch size by exploring architectures with different input sizes and evaluating segmentation performance on the validation set. For this optimization step, we trained the network for 100 epochs and quantified the performance by the Dice coefficient. Among ten

tested sizes (from [8 8 8] to [32 32 32]), we found the optimal patch size to be [16 16 12] voxels (x,y,z), with the maximum Dice coefficient being 0.48 on the validation data.

Using this patch size, we trained the final network for 500 epochs with a learning rate of 1e-4. To train SegVess, we randomly extracted 2000 patches of size 16 x 16 x 12 from the training set and further augmented this data by random rotations around the z-axis as well as horizontal flipping, which increased the size of the training data by a factor of 8 (16,000 patches in total). Finally, we tested the performance of the network using the reserved test set which was unseen by both EnhVess and SegVess during training.

To demonstrate the result of this network, we applied SegVess to the output of EnhVess on an OCTA image (of size 512 x 512 x 350 voxels) unseen by either network. The enhanced image was divided into patches of size 16 x 16 x 12 and each patch was processed by the network to produce the final segmentation.

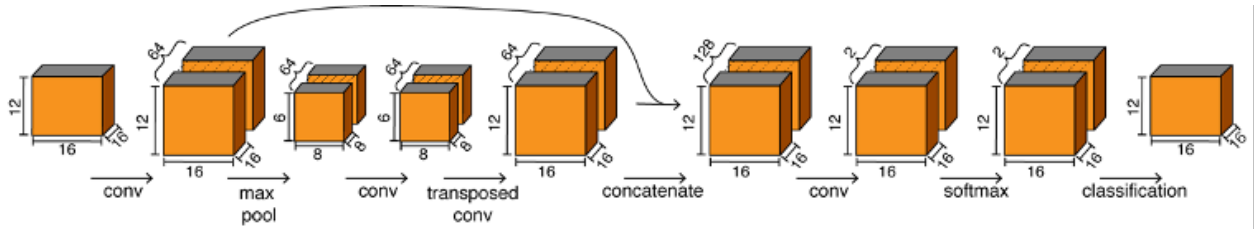


Figure 3.3. Architecture of SegVess used for volumetric vessel segmentation. The output of EnhVess is used as the input to SegVess.

3.3.6. Developing and training ConVess: Improving vessel connectivity in the segmented binary image

To correct any obvious gaps in the segmented binary image (the output of SegVess), we developed and trained another CNN, named ConVess. To obtain training data for ConVess, we corrupted our manual segmentation by randomly deleting small portions of the segmentation,

thereby introducing obvious gaps within vessel segments. ConVess was trained to efficiently correct these gaps using both the original and enhanced OCTAs. We opted for this approach as it makes use of the underlying experimental data, unlike other popular gap-correction methods like tensor voting. The structure of this CNN is identical to EnhVess (Fig. 3.2), but with 3 inputs: the corrupted segmentation, the standard OCTA, and the enhanced OCTA. These inputs are concatenated to produce a 3D image with 3 channels, such that the network could learn features from the gray-scale images on how to correctly connect gaps. We used the same ground truth data as EnhVess and SegVess, and the reserved data for testing the entire pipeline that was unseen by any of the three networks during training.

To train ConVess, we generated about 100 random gaps in the OCTA image ($200 \times 200 \times 230$ voxels) such that the center positions of the gaps were located within the segmented vessels. The gaps were cubic in shape, the size varied between $1 \times 1 \times 1$ voxels and $4 \times 4 \times 4$ voxels, and the edges were smoothed using a Gaussian filter. One-hundred and twenty patches of $32 \times 32 \times 32$ voxels were randomly obtained from this corrupted OCTA, and the dataset was augmented by random rotations around the z-axis as well as horizontal flipping, which increased the size of the training data by a factor of 8 (960 in total). Finally, we tested ConVess on another corrupted OCTA which was generated from the original, uncorrupted OCTA image by the same method.

The trained connectivity-enhancing network was then applied to the segmented image (the output of SegVess on the new, unseen OCTA) by splitting the image into $32 \times 32 \times 32$ patches and applying the network to each patch.

3.3.7. Vessel Graphing

Our strategy for graphing vessels consists of several steps applied to the output of ConVess. Firstly, we morphologically close the binary image using a $3 \times 3 \times 3$ kernel to close any further gaps that were not corrected by ConVess, and we remove any connected components smaller than 50 voxels.

This post-processed segmentation is then skeletonized using 3D medial axis thinning. This skeletonization converts the 3D binary segmentation image into a 3D binary image where all vessel-like structures are reduced to 1-voxel wide curved lines. To ensure that the curved line (“skeleton”) passes through the centerline of the vessel, we “recentered” the skeleton using a method inspired by Tsai et al. [33]. In detail, assuming that the intensity of vessels in the enhanced angiogram (the output of EnhVess) is brightest along the centerlines of the vessels, we analyze the local $3 \times 3 \times 3$ voxel neighborhood of each voxel of the skeleton, except for those voxels corresponding to endpoints. The skeleton is recentered by shifting voxels in a three-step hierarchical sequence: (1) A voxel is deleted if doing so does not change the local connectivity. The local connectivity herein was defined by the number of connected objects within a $3 \times 3 \times 3$ cube around the voxel. (2) If removal is not possible (i.e., if the removal changes the local connectivity), we move this voxel to an unoccupied location that must be within one voxel from the original location, must correspond to a higher intensity in the enhanced angiogram, and must not change the local connectivity. (3) If neither removal nor movement is possible, but if there is an unoccupied neighboring voxel with greater intensity in the enhanced angiogram, we activate that voxel. These steps are repeated for all centerline voxels and iterated until no further changes occur.

Next, we locate all bifurcations in the centerline, and describe each vessel segment as a list of voxel positions in 3D, referred to as edges here. We then extracted the cross-section of the

enhanced angiogram perpendicular to the direction of each edge at each voxel position, and fitted each cross-section to a 2D gaussian function, returning the correlation coefficient. The mean correlation coefficient was measured for each edge and served as a measure of how likely each edge represents a “true” vessel. We examine edges with mean correlation coefficients less than 0.5, starting with the edge with the smallest value. If the global connectivity of the skeleton remains unchanged when skeleton voxels are removed at the positions describing the edge, then both the edge and those corresponding skeleton voxels are permanently removed, otherwise the skeleton remains unchanged and the edge is not deleted. The global connectivity herein was defined by the number of endpoints within the skeleton. Finally, we successively remove branches shorter than 7 voxels in length, starting with the shortest branches. Once this process is complete, we manually inspect the results using a custom-made GUI which allows us to delete, create or connect vessel segments.

Any vessels sharing a branchpoint are considered to be connected, such that each vessel is connected to either 2 or 3 other vessels at each end, unless this vessel is at the boundary of the imaging volume in which case only one end of the vessel is connected to other vessels. Therefore, the final result of vessel vectorization is given by a collection of nodes (branching points) and the vessels connected to each node, where each vessel is described by a list of voxel positions in 3D.

3.3.8. Measurement of angio-architectural properties

The vessel tortuosity was calculated by dividing the vessel length by the straight-line distance between the vessel segment endpoints. To calculate the branching orders, we first identified penetrating vessels from the intensity (structural) OCTA, which appear as dark circles. Vessels which are connected to these penetrating vessels are considered “first-order vessels”. Then, we found the vessel segments connected directly to these vessels and classified them as “second-

order vessels” and so on until vessels with terminal endpoints were reached. To measure the vessel diameter, we first extracted the cross-sections of each vessel along the centerline and then fitted a 2D Gaussian to each cross-section. For each vessel, we chose only the cross-sections with the coefficient of determination (R^2) above 0.8 and averaged the full width at half maximum of these selected cross-sections.

3.4. Results

3.4.1. EnhVess: OCTA enhancement and “tail” removal

We evaluated the trained EnhVess by applying it to a new standard OCTA image that was unseen during training, and compared the result to common image enhancement techniques: rod filtering [33, 34], top-hat filtering [13, 35], Hessian-based filtering [36, 37], and optimally-oriented flux (OOF) enhancement [38, 39]. The enhancement methods were parameterized to provide the best results on capillaries. Rod filtering and top-hat filtering are accomplished by applying a rod filter of diameter and length of 2 voxels and 11 voxels respectively. For Hessian-based filtering and OOF, we chose a diameter range of 1 to 5 voxels in increments of 1 voxel. All methods enhanced visualization of microvessels when viewed from above (en-face projection), but the cross-sectional slices reveal significant distinctions between the methods (Fig. 3.4). The figure shows that with EnhVess the projection artifacts are significantly diminished, and the image is considerably denoised. The vessels maintain their tube-like shapes such that the vessel cross-sections appear circular instead of elongated as in the case of the other image enhancement techniques. This tail mitigation is further illustrated in Fig. 3.5. Among the other techniques, the Hessian-based filtering results in relatively better cross-sectional shapes of vessels, but the shape is still elongated in most vessels when compared to the result of EnhVess.

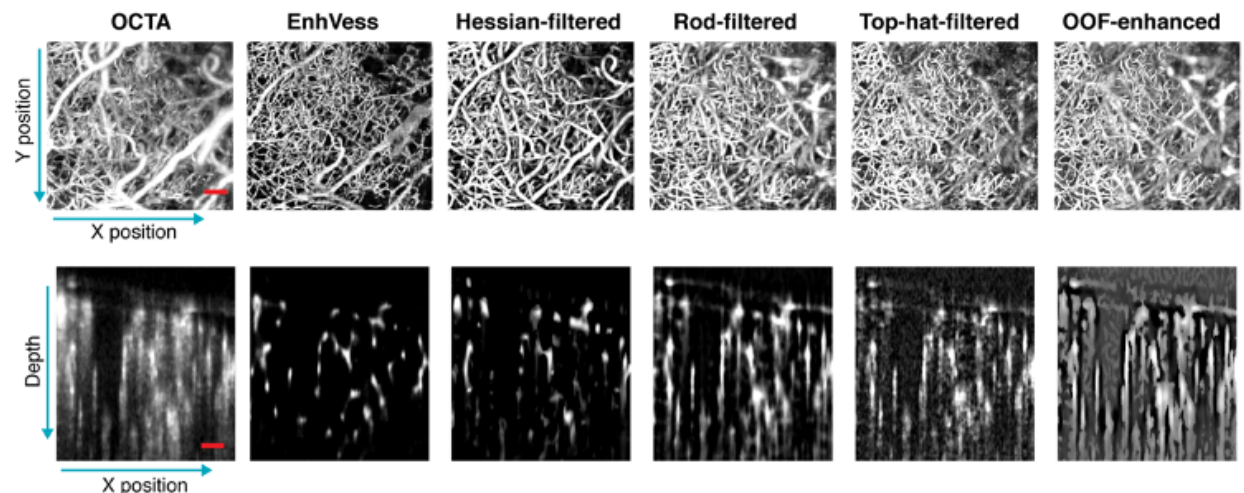


Figure 3.4. Comparison of EnhVess with common vessel enhancement techniques, viewed as the en-face maximum intensity projection (MIP) (top) and as the cross-sectional slice (bottom). Scale bars, 0.1 mm.

To assess the performance of EnhVess quantitatively, we compare common metrics of image quality, namely peak signal to noise ratio (PSNR), mean-squared error (MSE) and structural similarity index (SSIM) with respect to the segmented image [40, 41]. These metrics are calculated over B-scans and the mean and standard deviation is derived. Higher values for PSNR and SSIM, and lower values for MSE indicate better image quality. As shown in Table 1, EnhVess performs best according to all 3 metrics. All three image quality metrics indicate that every technique tested resulted in statistically significant enhancement compared to the original OCTA, and furthermore that EnhVess produced enhancement that was statistically better than every other enhancement technique as determined by one-way ANOVA.

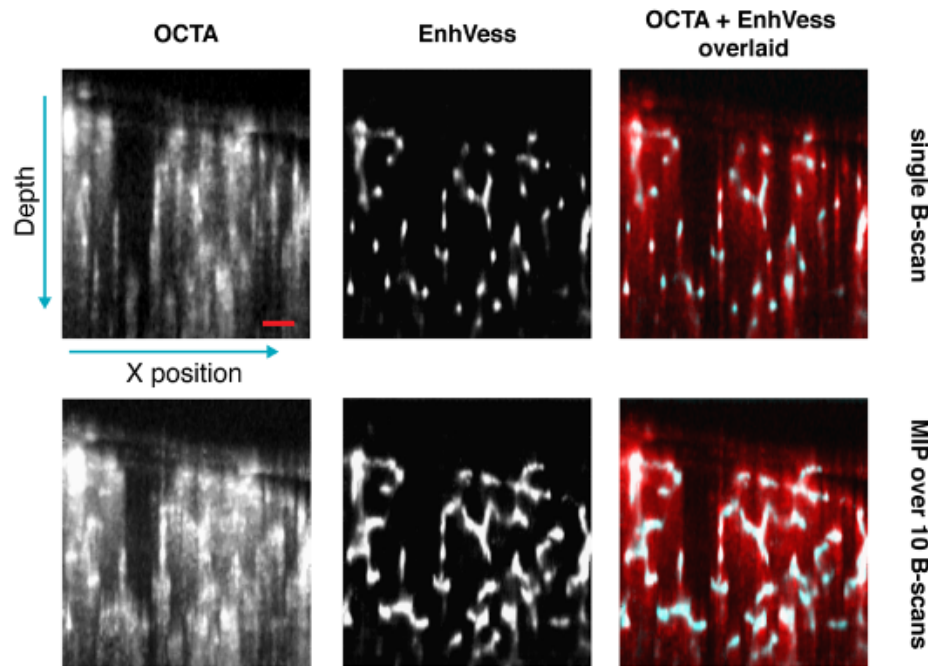


Figure 3.5. Demonstration of projection tail mitigation by overlaying the EnhVess result over the original OCTA. Top row shows a single B-scan, and bottom row shows MIP over 10 B-scans. Scale bar, 0.1 mm.

Table 3.1. Comparison of Image Enhancement Techniques

	PSNR	SSIM	MSE
OCTA	12.9 ± 0.8 (***)	0.013 ± 0.003 (***)	0.052 ± 0.010 (***)
EnhVess	19.2 ± 1.0 (***)	0.776 ± 0.041 (***)	0.013 ± 0.003 (***)
Hessian-filtered	16.5 ± 1.3	0.711 ± 0.045	0.023 ± 0.007
Rod-filtered	16.1 ± 1.2	0.408 ± 0.068	0.026 ± 0.008

Top-hat filtered	15.8 ± 1.2	0.056 ± 0.004	0.027 ± 0.008
OOF-enhanced	15.3 ± 1.4	0.818 ± 0.046	0.031 ± 0.010

*** significant at $p < 0.001$.

3.4.2. SegVess: three-dimensional OCTA segmentation

To evaluate SegVess, we used the data reserved for testing, which was unseen to EnhVess and SegVess during training (see Methods), along with the corresponding manual label. The test data was input to EnhVess first, and its output (enhanced OCTA) was input to SegVess. Qualitatively, we observed excellent agreement between the output of SegVess (segmented binary 3D image) and the manual segmentation (Fig. 3.6). Importantly, the tubularity of vessels was well preserved, implying that the projection artifacts present in the original angiogram were effectively removed by EnhVess and thus negligibly affected the segmentation.

To quantitatively assess the performance of SegVess, we used the area under the curve (AUC) of the receiver operating characteristic (ROC), a widely used measure of a model's capability of accurately classifying different classes. To determine the ROC curve of SegVess, we examined the output of the softmax layer at various thresholds and plotted the true positive rate against the false positive rate. It should be noted that the final segmentation is obtained by thresholding the softmax layer at 0.5, as the network was trained to optimize the probabilities of each voxel belonging to the correct class using the Dice coefficient. We compared the ROC curve of SegVess to those of the widely-used traditional thresholding methods, global and adaptive thresholding as applied to the standard angiogram. For global thresholding, the optimal threshold was determined by calculating the dice coefficient for a range of thresholds. For

adaptive thresholding, two parameters are generally chosen: the neighborhood size and the sensitivity. To compare SegVess to the best possible adaptive thresholding result, we chose the neighborhood size that yielded the best AUC score, although this AUC-based optimization is not feasible in the common situation where the ground truth is not given. Also, to further enhance the thresholding results, we removed connected components smaller than 10 voxels and applied gaussian filtering to smoothen the edges. As expected, the adaptive thresholding performed better than the global thresholding (Fig. 3.6).

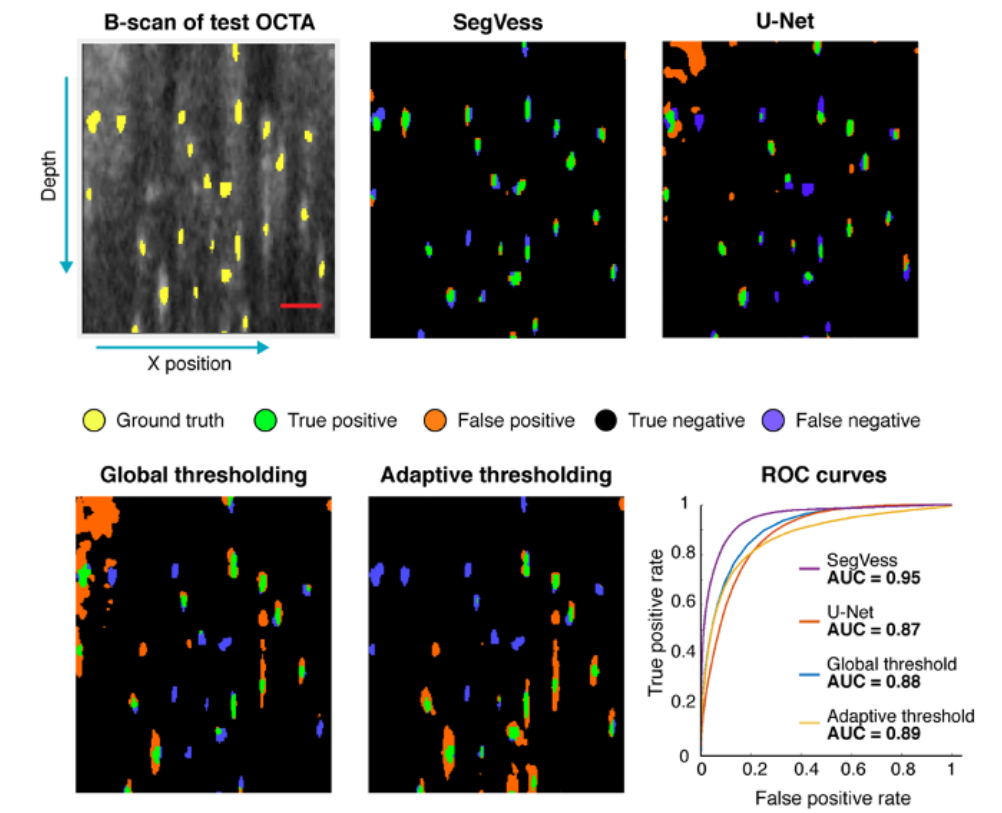


Figure 3.6. Comparison of SegVess with global thresholding, adaptive threshold and U-Net. A B-scan is presented out of the volume with 200 x 32 x 230 voxels (x, y, z). Ground truth is shown in yellow on the first OCTA image, and the true positive, false positive, true negative, and false negative are presented in the segmented images. Scale bar, 50 mm.

Compared to the combination of EnhVess and SegVess, however, the thresholding methods are susceptible to the projection artifacts, producing more false positives. Further, the

thresholding methods require the choice of optimal parameters while SegVess does not require any user parameters to be tuned. Lastly, we compared SegVess to U-Net [42] which was trained on the same data with the same patch size and found U-Net to perform similarly to global and adaptive thresholding. Since the U-Net has millions of parameters, we attempted to reduce overfitting by employing drop out layers with a probability of 0.5 after each maxpooling and upsampling layer.

After evaluation, we applied SegVess to the output of EnhVess shown in Fig. 3.5. Fig. 3.7 displays this segmentation result as an en-face MIP image and a 3D view of a part of the 3D binary image.

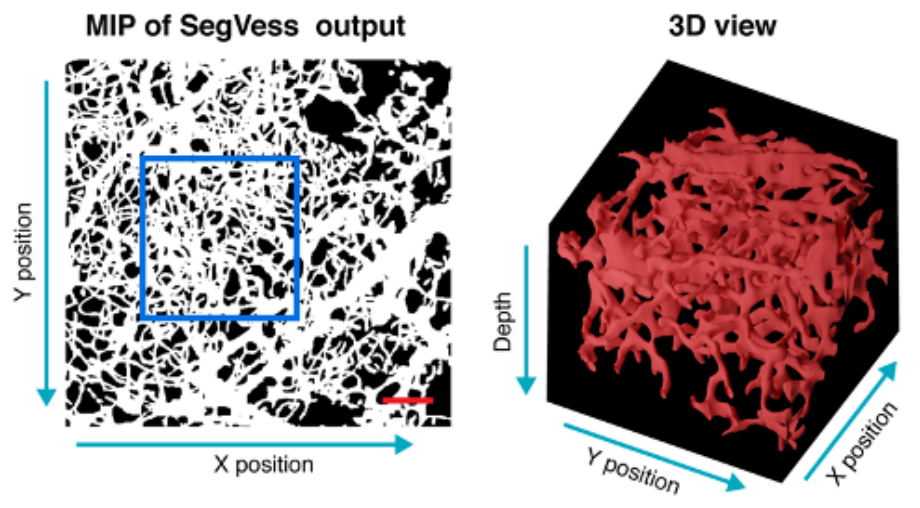


Figure 3.7. The output of SegVess. Scale bar, 0.1 mm. The 3D view box shows 0.3 mm x 0.3 mm x 0.5 mm (x, y, z).

3.4.3. Convess: Automated gap correction

Although the segmentation by SegVess is significantly better than the traditional thresholding methods, it is still prone to gaps either within a vessel or between vessels. Such gaps appearing in automated segmentations have been a major source of the need for labor-intensive manual correction in the later vessel-graphing step [43]. Machine-learning approaches other than deep learning were developed and tested to partially automate this manual correction. For example, Kaufhold et al. used a decision tree to identify good candidates for gap correction and spurious branch deletion so as to make the following manual inspection and correction less time-consuming [34]. Here, we used our third network ConVess to automatically identify and correct gaps present in the output of SegVess (the segmented 3D binary image). See Methods for details of the network and its training.

We tested the trained ConVess on an OCTA image with artificially created gaps: we assessed the extent of gap-correction by leveraging the fact that an uncorrected gap in the segmentation leads to a gap in the resulting skeleton. More specifically, we skeletonized the corrected segmentation (output of ConVess) as described in the Methods and examined the region around each of the artificial gaps in the skeleton to determine if the gap had been corrected. As a result, ConVess corrected 94% of the gaps. Figure 3.8a displays a few examples of the gaps appearing in the output of SegVess and how those gaps were corrected by ConVess. The MIP of the output of ConVess is presented in Fig. 3.8b.

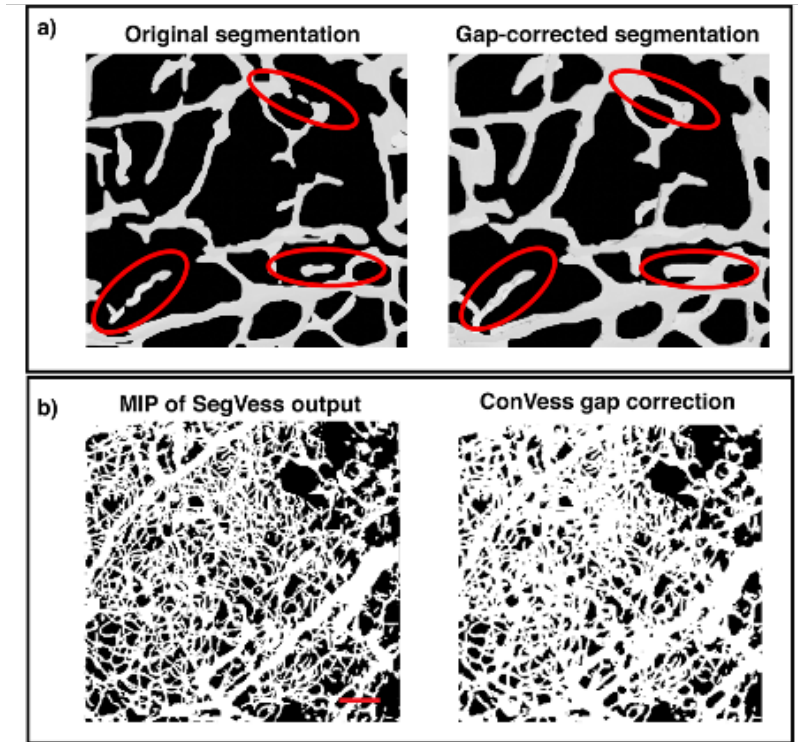


Figure 3.8. Results of ConVess. (a) Examples of how ConVess corrected gaps. (b) Input and output of ConVess in MIP. SegVess receives the output of SegVess as the input (left) and returns the gap-corrected, 3D binary vessel segmentation image as the output (right). Scale bar, 0.1 mm.

To quantitatively assess the performance of ConVess without artificially adding gaps, we applied ConVess to the output of SegVess shown in Fig. 3.6 which has the ground truth. ConVess improved the segmentation, as demonstrated by the metrics shown in Table 2. Importantly, the true positive rate increased significantly as well as the dice score. There was only a small change on the false positive rate, although this change is also statistically significant as determined by paired t-tests. The relatively low dice score might be attributed to the difficulties in defining vessel boundaries due to the nature of OCTA imaging. However, the low score would not be problematic in practice because most OCTA studies focus on relative diameters between control and experimental groups than the absolute value while most studies

measure the diameter from the gray-scale angiogram, not from the segmented angiogram (see Discussion for details).

Table 3.2. Quantitative Comparison of ConVess to SegVess only.

	True positive rate	False positive rate	True negative rate	False negative rate	Dice score
SegVess	0.47 ± 0.13	0.01 ± 0.01	0.99 ± 0.01	0.53 ± 0.13	0.52 ± 0.13
SegVess + ConVess	0.59 ± 0.13 (***)	0.02 ± 0.02 (***)	0.98 ± 0.01 (***)	0.41 ± 0.13 (***)	0.55 ± 0.13 (***)

*** significant at $p < 0.001$.

3.4.4. Graphing the vasculature network and extracting angioarchitectural properties

The gap-corrected, 3D binary vessel segmentation image underwent several processing steps to produce a vascular network graph, including skeletonization, spurious branch detection, and edge re-centering. Figure 3.9a displays the graph obtained from the gap-corrected segmentation shown in Fig. 3.8.

This representation of a vascular network as a graph allows various properties of the angioarchitecture to be easily quantified in an objective manner. In this paper, we demonstrate measurements of branching orders (Fig. 3.9a), vessel lengths (Fig. 3.9b), vessel diameters (Fig. 3.9c), and vessel tortuosity (Fig. 3.9d).

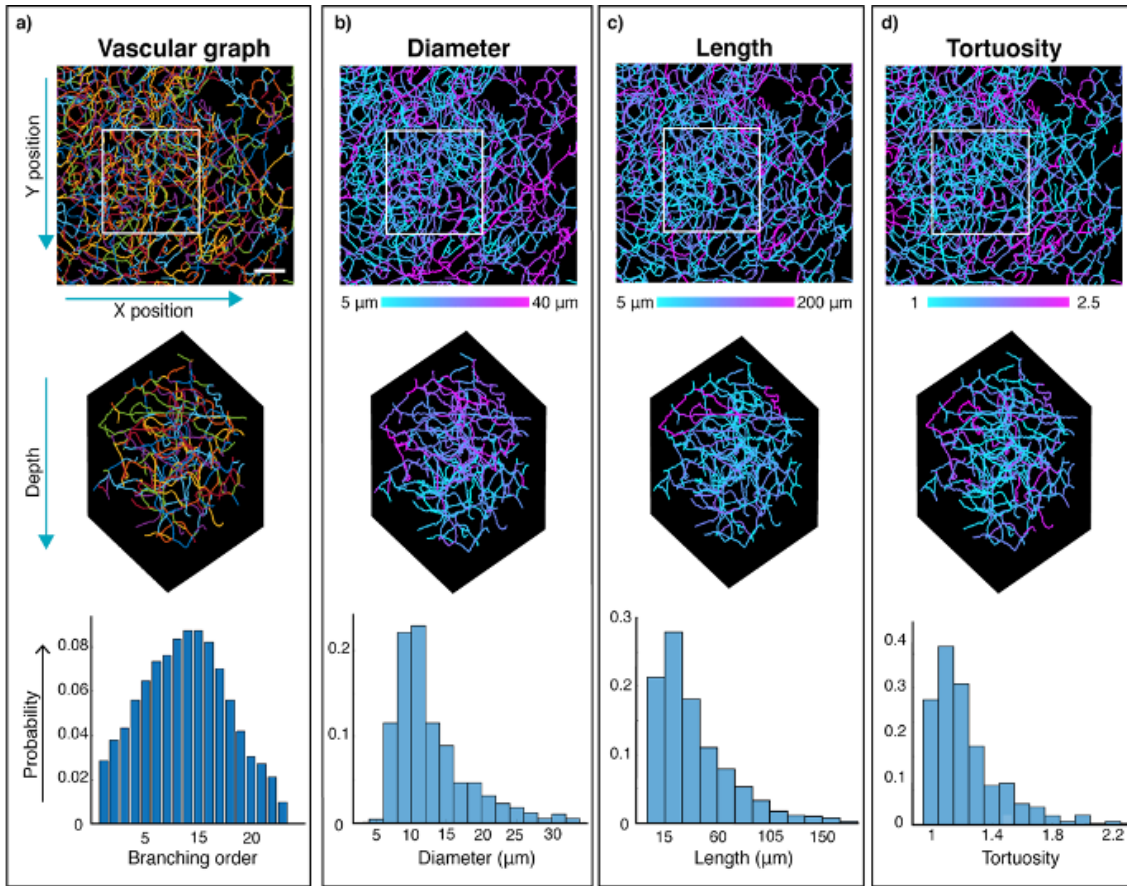


Figure 3.9. Application of CNNs to real microvascular data, and quantification of vascular properties. **a)** The result of graphing the vessels from the output of ConVess. The top row shows the *en-face* projection of the vectorized vasculature, where each vessel is displayed with a random color. The middle row displays a 3D view of the region indicated by the white square. The bottom row presents the distribution of vessels in the top row by branching order. **b)** Each vessel is shown colored according to the mean diameter as averaged along the vessel axis. The histogram in the bottom row shows the distribution of vessels by diameter. **c)** Each vessel is shown colored according to the length of the vessel. The histogram in the bottom row shows the distribution of vessels by length. **d)** Each vessel is shown colored according to tortuosity of the vessel. The histogram in the bottom row shows the distribution of vessels by tortuosity. Scale bar, 0.1 mm.

Figure 3.10a summarizes the pipeline presented in this paper, from an OCTA to EnhVess, to SegVess and ConVess, and finally to a vascular graph with associated angioarchitectural properties. This pipeline has been shown to be suitable for images obtained with different resolutions (Fig. 3.10b) and even for images obtained by other labs using a different OCT setup (Fig. 3.10c). The histograms of the angioarchitectural properties for each of these images were

similar among these datasets (Fig. 3.10d). The similarity, which should be true when animals were in the physiological condition, supports the generalizability of the developed approach to OCTA images obtained by other researchers.

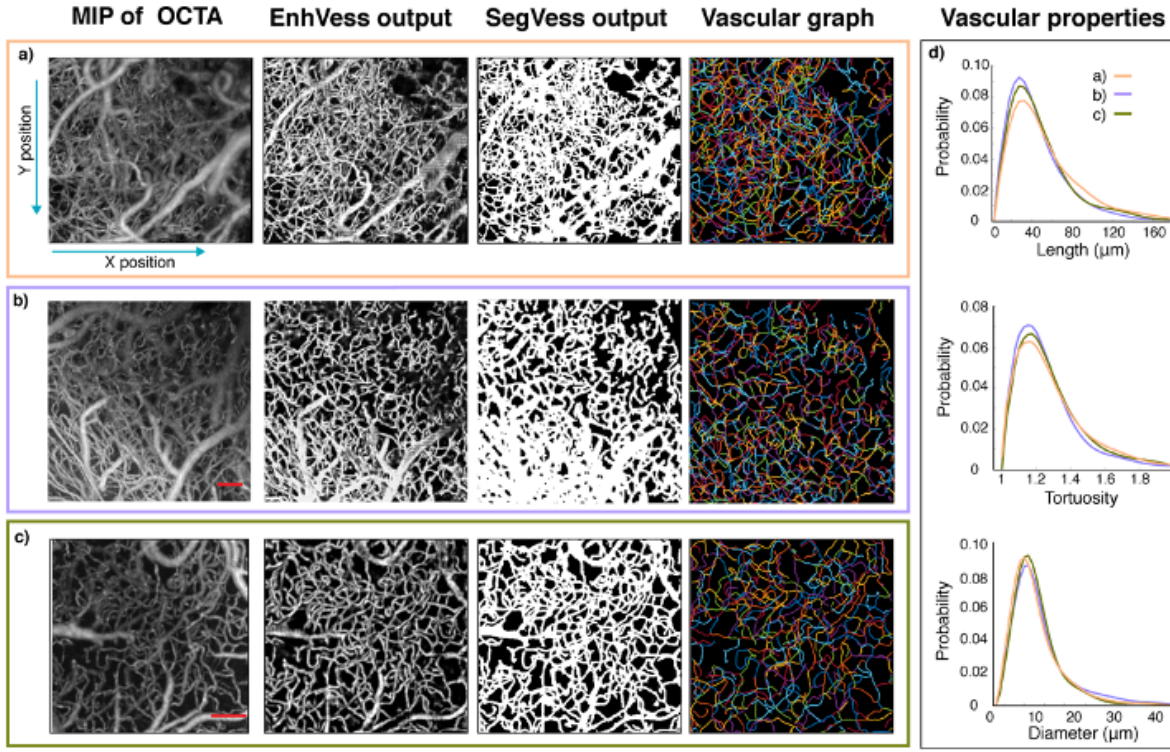


Figure 3.10. The processing pipeline of OCTA images with examples of the OCTA used in the previous figures (1.5- μm sampling rate) **a)**, an OCTA with a different sampling rate (3 μm) as obtained in our lab **b)**, and an OCTA obtained by a different lab **c)**. **d)** The histograms of vessel lengths, diameters and tortuosity extracted from each example. Scale bar, 0.1mm.

3.5. Discussion and conclusion

We have presented the pipeline of image processing procedures based on deep learning for the enhancement and segmentation of 3D OCTA images, and subsequent vascular graphing and quantification of angioarchitectural properties. The enhancement and segmentation are achieved using three CNNs: EnhVess, SegVess and ConVess.

EnhVess was designed for image enhancement based on the simple encoder-decoder architecture and was trained using an intrinsic-contrast “standard” 3D OCTA (input) and its manual segmentation (ground truth). This CNN significantly improved the signal-to-noise ratio and more importantly has shown to effectively suppress the OCTA-specific projection artifacts or tails caused by multiple scattering, restoring the tubular structure of vessels. EnhVess performed significantly better in this matter than other image enhancement techniques such as Hessian-based filtering, rod filtering, top-hat filtering, and OOF. These common vessel enhancement techniques were not effective in removing the projection artifacts, a unique challenge of OCTA. Because of these tails, researchers are often limited to analyzing 2D projections only, as it is challenging to distinguish vessels from background in cross-sectional views. The connectivity between vessels is also challenging to ascertain in cross-sectional views, hindering the accurate segmentation and vessel-graphing of cortical OCTA in 3D. EnhVess, as designed and trained specifically for cortical OCTA, improves vessel contrast and thereby enables the following processes to produce more accurate segmentation and graphing of the vasculature, which are essential for quantitative angioarchitecture analysis.

SegVess was designed for image segmentation based on 3D convolutional layers based on an architecture with fewer learnable parameters than popular segmentation networks like U-Net. SegVess was trained on the output of EnhVess (input) and the manual segmentation (ground truth). SegVess achieved an AUC score of 0.95 while global and adaptive thresholding-based segmentations scored 0.88 and 0.89, respectively, and the U-Net scored 0.87. The distinction was made clearer when assessing the tubularity of vessels in the cross-sectional views. SegVess thus accomplishes automated segmentation of 3D OCTA images with the rigorous AUC score, which is imperative from a practical perspective. While manual segmentation remains the gold standard, it is a subjective, laborious, and time-consuming task which is generally not feasible for large datasets. In this study, the manual segmentation took place over

the course of three months for the image of $300 \times 300 \times 350 \text{ um}^3$ or $200 \times 200 \times 230$ voxels (x, y, z). In addition to this long time required, the manual segmentation required an external contrast agent for high confidence in the result, together making manual segmentation impractical for research like our ongoing study that analyzes many OCTA volumes. In contrast, SegVess only takes several seconds and works well without the aid of extrinsic contrast agents. It should be noted that the Intralipid-contrast OCTA had not been used in training any CNN in this paper but was only consulted to guide manual segmentation.

The use of the extrinsic contrast agent was critical in visualizing vertical capillaries for the guidance, because the OCTA contrast depends on the angular orientation of a capillary vessel [44]. It is a known challenge to visualize vertical capillaries in intrinsic-contrast OCTA images, but Intralipid improves this visualization (Figs. 3.11a and 3.11b). We also note that EnhVess is capable of enhancing the visibility of these vertical capillaries without the use of any extrinsic contrast agent (Fig. 3.11c).

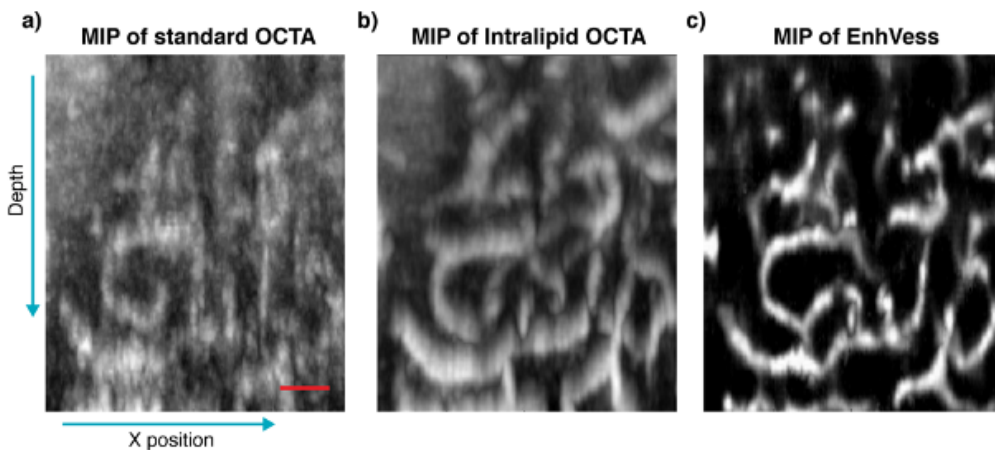


Figure 3.11. Enhancement of vertical capillaries using EnhVess. **a)** MIP of intrinsic-contrast OCTA image over 32 B-scans. **b)** MIP of registered Intralipid-contrast OCTA. **c)** MIP of the EnhVess output in response to the input of the intrinsic-contrast OCTA. The Intralipid OCTA **b)** was not used in training EnhVess. Scale bar, 50 μm .

ConVess was designed for further improvement of the segmentation, especially with respect to gap correction, based on the same architecture as EnhVess but with 3 input channels. The input consists of the segmentation (output of SegVess) along with the enhanced image (output of EnhVess) and the original OCTA image. We found that the gap-correction is more accurately done when it involves the third input so as to be guided by the grayscale data. This CNN identified and corrected 94% of gaps on the validation data. When applied to the test data, ConVess resulted in fewer gaps in the vascular graph and therefore less manual effort for correcting errors in the following steps in the pipeline.

Finally, the pipeline extracts a network graph representing the vasculature from the gap-corrected, segmented angiogram through moderately improved processes for pruning, loop removal, and re-centering. Such a graph enables the measurement of various angioarchitectural properties, including but not limited to the vessel length, diameter, tortuosity, and branching order. The distributions resulting from our pipeline (Fig. 3.10) closely resemble those reported using two-photon microscopy in terms of shape as well as value ranges [30]. The distributions shown in Fig. 3.10 are also similar in shape, as expected for healthy, wild-type mice.

The values of diameters obtained, however, may be inaccurate due to the mechanism of OCTA contrast. The intrinsic contrast of OCTA originates from the movement of RBCs through vessels. This may result in variations in diameter along the centerline of a single vessel, especially in capillaries where RBCs move one by one. The contrast based on RBC movement also makes it unclear which point in the cross-section of a vessel in OCTA exactly indicates the vessel wall. Thus, we measured the diameter by averaging the cross-sections along the centerline, which makes the measurement precise against the variations in diameter. But it is still not accurate depending on the definition of the vessel diameter, due to the uncertainty regarding the vessel wall. Despite this limitation in accuracy, high precision is often sufficient for

many studies that focus on relative differences between experimental conditions, rather than absolute diameters.

We have made all of our algorithms in the presented pipeline freely available as a MATLAB toolbox for immediate use https://github.com/sstefan01/OCTA_microangiogram_properties. This toolbox has been implemented to be as user-friendly as possible, such that researchers may simply input an OCTA image and then receive the vascular network graph detailing the connectivity between vessels, as well as the associated angioarchitectural properties of every vessel. We hope that the user-friendly toolbox promotes the utilization of OCTA as a full 3D method for analysis of microvasculature, eventually unlocking the full potential of the imaging tool for neuroscientific research.

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3.8. Disclosures

The authors declare no conflicts of interest.

3.9. References

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4. Deep Learning and Simulation for the Estimation of Red Blood Cell Flux with Optical Coherence Tomography

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4.1. Abstract

We present a deep learning and simulation-based method to measure cortical capillary red blood cell (RBC) flux using Optical Coherence Tomography (OCT). This method is more accurate than the traditional peak-counting method and avoids any user parametrization, such as a threshold choice. We used data that was simultaneously acquired using OCT and two-photon microscopy to uncover the distribution of parameters governing the height, width, and inter-peak time of peaks in OCT intensity associated with the passage of RBCs. This allowed us

to simulate thousands of time-series examples for different flux values and signal-to-noise ratios, which we then used to train a 1D convolutional neural network (CNN). The trained CNN enabled robust measurement of RBC flux across the entire network of hundreds of capillaries.

4.2. Introduction

The microvascular bed is integral to the delivery of nutrients and oxygen to cerebral tissue, and its dysfunction is implicated in a number of pathologies. To understand how various experimental conditions impact microvascular flow, it is critical to be able to study a large number of capillaries considering the heterogeneity of flow in capillaries. The ability to study blood flow in a network of interconnected capillaries makes it possible to test a wide range of scientific hypotheses on how microvascular flow is regulated in the brain in health and disease. For example, microvascular flow has been studied in relation to neurovascular coupling [1], stroke [2] and Alzheimer's disease [3].

However, it is not currently feasible to accomplish high-throughput flow measurements using two-photon excitation fluorescence microscopy (TPEF), the gold standard for quantitative microvascular imaging [4–6]. As an alternative, optical coherence tomography (OCT) has shown promising results for the quantification of blood flow, as demonstrated by Doppler OCT for imaging arterioles and venules [7,8]. Doppler OCT has also shown some promise in quantifying red blood cell (RBC) speed [9] although RBC flux may be more physiologically relevant as it more closely correlates with oxygenation [10]. However, in comparison to larger vessels, the dynamics of blood flow differ greatly in capillaries which are typically smaller than 10 μm and in which RBCs flow discretely in single file, making it difficult to apply Doppler OCT to determine RBC flux. This is because the fast A-scan rate defining Doppler OCT results in only 1–2 RBC passages being observed during a reasonable acquisition period, which is insufficient to

determine RBC flux. Instead, OCT has been leveraged to quantify RBC flux in large networks of capillaries, based on the observation that a passage of an RBC through a voxel causes a transient increase in intensity [11,12]. The standard approach is to count the number of peaks observed over a length of time using some peak-finding algorithm, commonly based on a threshold: i.e., peaks over this threshold are considered to be peaks resulting from RBC passages [13]. However, this approach is sensitive to noise, requires user parametrization, and has been shown to underestimate RBC flux at high flux values (over 80 RBC/s) [14]. Often, it is difficult to manually distinguish noise from legitimate RBC passages, making it difficult to assess whether a peak-counting algorithm is working correctly or whether the threshold is appropriate.

To address this issue, we aimed to develop a robust and accurate method of determining RBC flux from OCT data which does not require any user parametrization. We exploited deep learning to extract relevant features from OCT time-series data using a 1D convolutional neural network (CNN). To train the network, we collected RBC passage data using OCT and TPEF simultaneously, where the TPEF data provided the true flux value. We probed the CNN to understand which features were important for classification of RBC flux, and then incorporated these results to simulate many OCT time-traces for different RBC flux and velocity values. This enabled us to produce hundreds of thousands of training examples, which is especially important for low and high flux values which are underrepresented in real data. The result is a validated method for simulating OCT time-series data, and a CNN that can be implemented to obtain accurate and robust estimations of RBC flux in large networks of capillaries.

4.3. Dataset

As described by Marchand et al. [14], a multi-modal platform was designed for simultaneous in vivo imaging using OCT and TPEF. TPEF imaging was performed at 920 nm and FITC

fluorescence was collected through an emission filtered at 520 nm. An objective lens with magnification of 10× (Mitutoyo) was used to provide a lateral resolution <3 μm for TPEF imaging, and 3.5 μm for OCT. The light source of the OCT system operated at 1300 nm (LS2000C, Thorlabs) with an axial resolution of ~ 3.8 μm in animal tissue.

For in vivo imaging of the cerebral cortices of C57BL/6 mice, we performed craniotomies with approval from the ethics committee of the research center of the Montreal Heart Institute. For the craniotomy performed at Brown University, experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Brown University and Rhode Island Hospital. Cranial windows were installed to gain optical access to the cerebral cortex, according to the widely adopted protocol described by Mostany and Portera-Cailliau [15].

To obtain the simultaneous time-courses using OCT and TPEF, we performed imaging in the awake state. Firstly, we selected a capillary through the TPEF channel, since only a single capillary can be analyzed at a time using TPEF. Line-scanning was performed at a rate of 800 Hz along the longitudinal axis of the capillary for approximately 60 s along a line of 11.7 μm. At the same time, B-scans were acquired using the OCT to capture the intensity time-course of the same vessel. These B-scans were repeated over a time of 0.768 s at a rate of 665 Hz, resulting in a temporal resolution of 1.5 ms. Each B-scan consisted of 60 × 2048 pixels, spanning 100 μm laterally, resulting in a lateral pixel size of 1.7 μm. The TPEF time-course was then downsampled to the same frequency as the OCT. Overall, 9441 time-courses of RBC passages were obtained from a total of 3 mice and 119 capillaries.

We performed additional OCT imaging to demonstrate our method on a large network of capillaries, which we performed using a commercial SD-OCT system (Telesto III, Thorlabs) with center wavelength of 1310 nm and bandwidth of 170 nm. This imaging was performed on one

C57BL/6 mouse under 2% isoflurane anesthesia. As before, we used a 10× objective lens (Mitutoyo) with transverse resolution of 3.5 μm . To image the network of capillaries, we scanned a region with a field of view of $256 \times 256 \times 70$ pixels (x, y, z) corresponding to a size of $768 \times 768 \times 245 \mu\text{m}$ (x, y, z). We did so by repeatedly performing B-scans at a rate of 714 Hz at each y position for over a time of 0.72 s, yielding a temporal resolution of 1.4 ms. We believe these imaging parameters to be appropriate for the intended purpose of measuring RBC flux in a network of capillaries, because the resolution is sufficient to observe individual capillaries and individual passages of RBCs, yet also balances the trade-off between resolution, dataset size, and acquisition time.

4.4. Methods

4.4.1. Training 1D Convolutional Neural Network on Experimental Data

We acquired 9441 time-traces of RBC passages using OCT and TPEF as described by Marchand et al. [14] consisting of 512 time-points at a temporal resolution of 1.5 ms. TPEF is often regarded as the gold standard for RBC flux measurements, since the TPEF time-traces exhibit high signal-to-noise ratio and are not plagued by speckle noise like the OCT data, allowing us to obtain the “ground truth” for the RBC flux. To confirm the higher signal-to-noise ratio, we determined the standard deviation of the normalized OCT and TPEF time-courses, and found a statistically higher signal-to-noise ratio as defined by the coefficient of variation for the TPEF time-courses ($p < 0.001$; paired t-test). Thus, we trained a 1D CNN, using the OCT time-traces as the training data and the RBC flux values obtained using TPEF as the labels. We augmented this dataset by reversing the time of the time-traces, and then split the augmented dataset into training, validation and testing with proportions of 70, 20, and 10%, respectively.

We adopted the state-of-the-art architecture for time-series classification, InceptionTime [16]. The CNN consists of an input layer of size 512 (corresponding to 0.75 ms), 3 inception modules, a global averaging layer, and a fully-connected layer which results in 2 outputs: the RBC flux prediction and its associated uncertainty. The inception module consists of a bottleneck layer, which feeds into 3 convolutional layers of kernel size of 10, 20, and 40. At the same time, the input of the inception module is also fed through a max-pooling layer of kernel size 3, and then a bottleneck layer. The output of the bottleneck layer and the convolutional layers are concatenated channel-wise and form the input of the next inception module.

We designed our CNN to provide an estimate of uncertainty in its prediction, which serves as a signal that a prediction may not be trusted. This is important because there is ambiguity in what constitutes the passage of a RBC, even when assessing the data manually. To provide a measure of uncertainty we adapted the loss function as shown in Eq. 1 to maximize the probability of a given prediction y , assuming this prediction is derived from a normal probability distribution with mean equal to the ground truth, G , and standard deviation, σ . The standard deviation, which is learned by the network, thus serves as an indicator of uncertainty in the predicted value. The network thus provides a continuous prediction for RBC flux, along with its continuous predicted uncertainty. We trained our network using the Adam optimizer, with a learning rate of $8e-4$ for 50 epochs, and a gradient threshold of 500 which was necessary given our adapted loss function.

$$loss = -\log \left(\frac{1}{\sigma\sqrt{2\pi}} \exp \left(\frac{(G-\hat{y})^2}{2\sigma^2} \right) \right) \quad (4.1)$$

We tested the performance of the CNN on the test data, which was unseen to the CNN during training, and observed more accurate results compared to peak-counting as assessed by the

slope being closer to 1, and the R^2 value higher (Figure 4.1). Therefore, the CNN provides the possibility to filter the predictions to include data with the highest confidence. We then tested the same network architecture trained on different input sizes to understand the trade-off between acquisition time and accuracy (Figure 4.1). It is important to note that increasing the acquisition time does improve the R^2 value of the peak-finding estimations, the slope is consistently less than 1, indicating a systematic bias against high-flux capillaries. On the other hand, the CNN can provide accurate predictions even for short acquisition times. We also tested this loss function in comparison to a conventional mean-squared loss, which does not provide a measure of uncertainty in its prediction. To do so, we trained a separate CNN with this mean-squared loss to perform regression on the same dataset as previously, and found that these results were still better than traditional peak-fitting (slope = 0.98, $R^2 = 0.85$), although it is not possible to weight predictions based on uncertainty like afforded by the loss function described in Eq. 4.1.

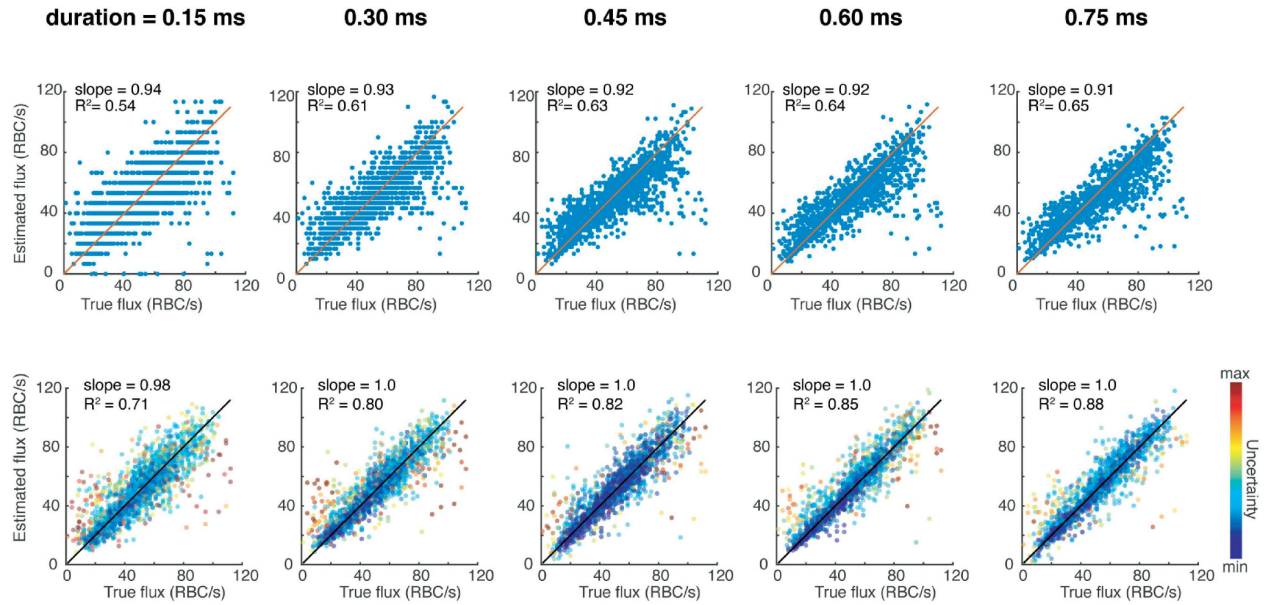


Figure 4.1. Red blood cell flux estimation accuracy as a function of acquisition time for the traditional peak counting method (top row) versus the presented deep learning-based method (bottom row).

4.4.2. Simulating Optical Coherence Tomography Time-Series Data

A significant benefit of knowing the ground truth for flux values is to enable determination of the underlying distributions of parameters describing the OCT time-series data: the shape, width, height and spacing of the peaks. We firstly identified likely RBC passages within a time-trace by identifying all peaks, sorting these peaks by peak prominence, and selecting the number of peaks corresponding to the true flux in order of descending peak prominence (Figure 4.2A). We used these peaks to assess the distributions of the parameters. We found that normalized time-traces with flux values between 20 and 80 RBC/s had peaks which had mean heights closer to 1 after normalization of each time-trace (Figure 4.2B), indicating that these peaks are likely to be detected by the traditional threshold-based methods. However, time-traces with higher flux values tend to have more peaks with smaller heights which may explain why the traditional methods tend to severely underestimate high RBC flux passages. This highlights the inherent difficulty in choosing an appropriate threshold to be sensitive to capture RBC passages in high flux capillaries while minimizing false positives in low flux capillaries. After having characterized the distribution of mean peak heights for each flux, we sought to describe the distribution of peak heights for a given flux and mean peak height within each time-trace, as shown in Figure 4.2E. For example, we find the mean peak height across all time-traces for a given flux is described by a beta distribution. Now, for a given mean peak height and flux, the height of intensity peaks within those time-traces are also described by a beta distribution, but with different parameters. Thus, for each flux and mean peak height, there is a unique beta distribution describing the peak heights within each time-trace, which is also true for peak width.

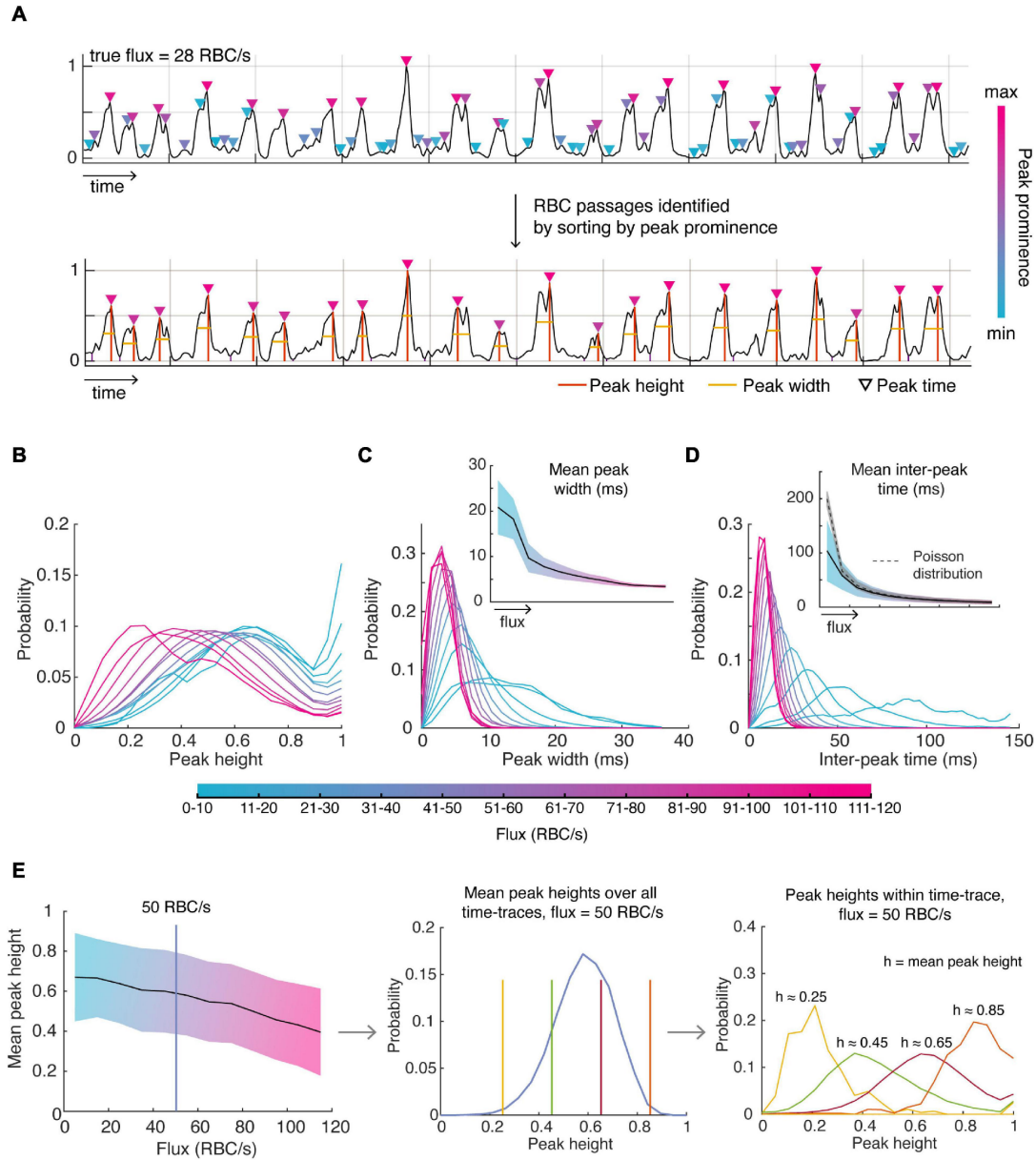


Figure 4.2. Detecting RBC passages from OCT time-series data and characterizing the distributions of parameters describing these RBC passages. (A) RBC passages were identified using the known value of flux (obtained by TPEF) and sorting peaks based on peak prominence. Orange lines describe peak position and heights, and the yellow line is the full width half maximum (FWHM), describing the width. (B) The distribution of all peak heights (C) peak widths and (D) inter-peak times for varying ranges of flux values. (E) Mean peak height decreases with increasing flux (left). Mean peak height over all time-traces for each flux category is described by a beta distribution (middle) as shown using 50 RBC/s as an example, and the peak heights within a time-trace given the mean peak height are also described by the beta distribution (right). Shaded areas show the standard deviation about the mean.

As expected, lower flux values are associated with larger peak widths considering the relationship between peak width and RBC speed (Figure 4.2C). The mean peak width for each flux value was well captured by the gamma distribution. For a given mean width, the gamma distribution also described well the distribution of peak widths within a time-trace. We also verify that the standard deviation of the average peak width decreases with increasing flux since large flux values can only be accomplished with higher RBC speeds (due to the finite size of the RBC) whereas low flux can be accomplished with low or high-speed RBCs. We also assessed the relationship between mean peak width and peak width variability, especially in the context of RBC speed, which was approximated as the reciprocal of the peak width. For example, we tested whether low RBC speed time-traces (i.e., high mean peak width) was also associated with greater variability in RBC speed, but we did not observe any relationships between mean RBC speed and RBC speed variability.

Next, we looked to describe the spacing between RBC peaks, or the inter-peak time (Figure 4.2D). As expected, the mean inter-peak time is inversely proportional to the flux – only the lowest flux category (<10 RBC/s) vastly deviated from this relationship by exhibiting lower mean inter-peak time than expected, likely an artifact due to the small number of RBCs observed during duration of each time-trace. We expected the distribution of inter-peak distances to follow a Poisson distribution with mean equal to the mean inter-peak distance. However, we found that the experimental standard deviation was greater for low flux values than predicted by a Poisson distribution (i.e., greater than the square root of the mean), only approaching a Poisson distribution for high flux values. We believe that this is due to the fact that low flux can be accomplished using a wide range of speed and hematocrit values (i.e. high speed and low hematocrit, or low speed and high hematocrit) whereas high flux values can only be accomplished by high speed and high hematocrit values.

Having characterized the parameter distributions describing how RBC passages appear in OCT data across all flux ranges, we were able to use this information to simulate RBC passages computationally for OCT imaging specifications similar to those described in the section Dataset. This will allow us to produce many more training examples of low and high flux time-traces for further training of our CNN, which were underrepresented in the experimental data. This also allowed us to produce many more training examples of rare but physiological instances, such as low-flux, high-speed time-traces. We first replicated our experimental data by simulating RBC passages as Gaussian pulses with identical mean height, width, and inter-peak distances. We then had the CNN of Figure 4.1 predict flux values from this set of noiseless, simulated training time-traces. Importantly, we discovered that the CNN overestimated low flux values and underestimated high flux values, implying that noise is an essential feature for the prediction of RBC flux.

4.4.3. Improving Optical Coherence Tomography Time-Series Simulations

We hypothesized that OCT-specific noise and slow-varying components are critical features that were omitted in the simple simulation above. Thus, we modeled OCT-specific noise and added the noise as well as slow-varying components, and then tested if the improved simulation improves prediction by the CNN.

We modeled OCT-specific noise as described in Uribe-Patarroyo et al. [17]. Here noise, N , is a random variable described by a zero-mean complex circular Gaussian distribution, which will be added to the signal, S , like so,

$$I = S + N^2 + 2\sqrt{S}|N| \cos \phi \quad (4.2)$$

Where ϕ is the relative phase between S and N and is modeled by a uniform distribution. Before incorporating noise into our simulations, we first tested whether the noise would affect the width of the simulated peaks. In detail, we added noise to the noiseless, simulated signals described above, while varying the amplitude of noise to achieve a range of signal-to-noise ratios from 1 to 10. We applied a gaussian filter with $\sigma = 1.5$ ms, while taking into account the effect of filtering on peak width. As expected, increasing noise resulted in an increased peak width, eventually reaching a plateau (Figure 4.3B). Furthermore, the fractional increase in peak width was independent of the original peak width. This corruption of peak width by noise may help explain the inaccuracy of estimation of RBC speed using OCT time-traces [14].

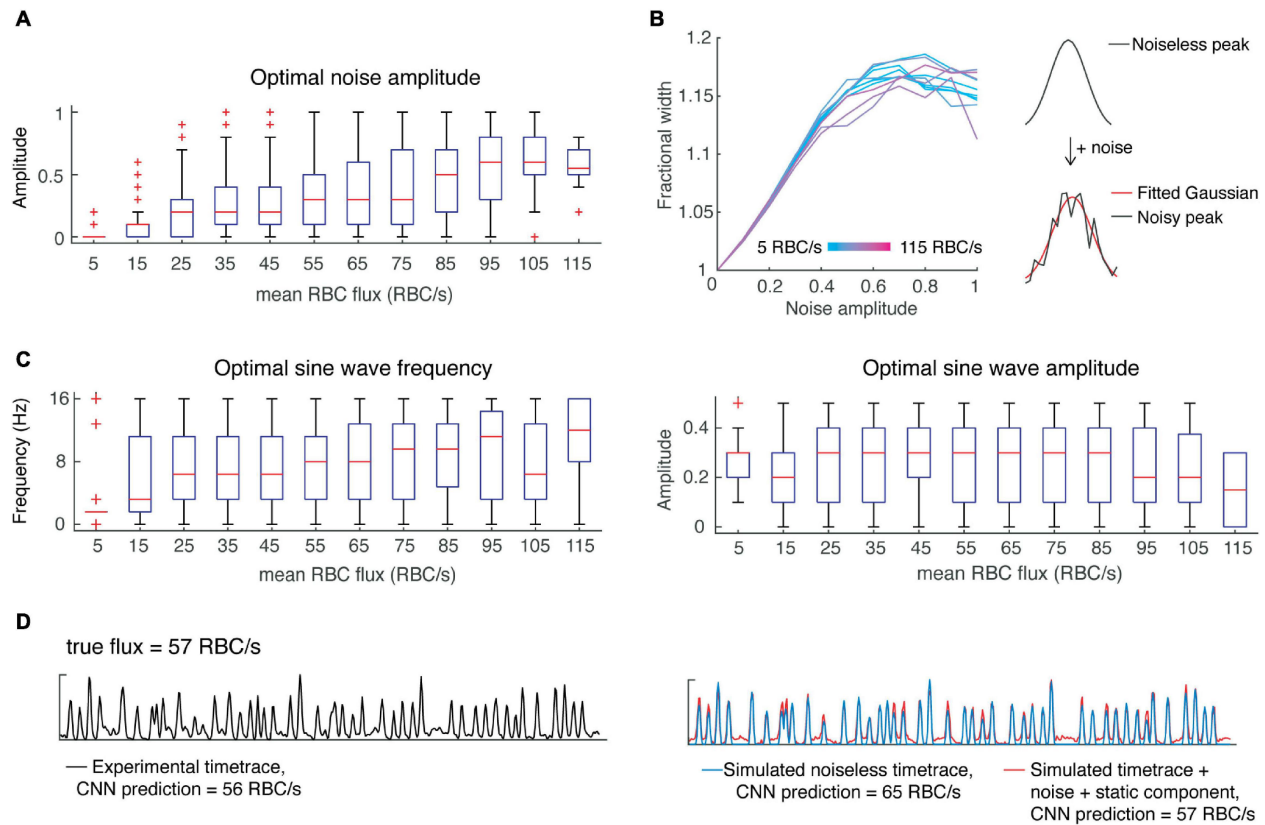


Figure 4.3. Incorporating noise and a slow-varying component improves predictions of CNN on simulated data. **(A)** Optimal noise amplitudes to maximize CNN prediction accuracy as a function of flux. **(B)** Including noise has the effect of increasing peak width as a function of the noise amplitude, reaching a plateau at around 17%. **(C)** Low flux value time-traces are predicted with greater accuracy when including a slowly-varying component with low frequency, whereas

all other flux values show greater variability in the optimal parameters of the slow-varying component. **(D)** An example showing how incorporating noise and a slow-varying component results in more accurate CNN predictions.

Next, we wanted to investigate whether noise amplitude was flux-dependent in the experimental data, and whether adding noise could improve the CNN predictions of our simulated data – i.e., is noise a critical feature needed for the classification of flux by our CNN? To answer these questions, we performed a grid search over different noise amplitudes (SNR ranging from 1 to 10) while correcting for the effect of noise on peak width. We found the optimal noise amplitude for each time-trace and assessed these results as a function of flux (Figure 4.3A). For high flux values (>100 RBC/s), we found better predictions were obtained with greater noise amplitudes, whereas for mid-range fluxes (~ 50 – 100 RBC/s), a wide range of noise amplitudes yielded optimal predictions. On the other hand, predictions of low flux values were almost always hindered by the addition of noise. This result suggests that there is a positive correlation between RBC flux and noise, which should be taken into account when designing algorithms for the simulation of OCT time-traces and the estimation of RBC flux. Another benefit of adding noise to the training data is the reduction of overfitting, i.e. the improvement of the generalization of the model, as has been demonstrated in a number of applications [18-20]. In fact, training with noise has been shown to be equivalent to weight regularization in which an extra term is added to the loss function [21].

We simulated noisy data using the average optimal noise amplitude for each flux range and verified that the number of peaks detected (using the previously determined peak prominence) was the same before and after the addition of noise. The CNN predictions from this noisy simulated data were improved in comparison to those from the noiseless data, except for very low flux values (<15 RBC/s) which were overestimated.

In addition to noise, OCT time-series can include a slow-varying component due to static scattering [22]. Therefore, we tested whether including a slow-varying component would improve the CNN predictions in a similar manner. We simply modeled this component as a sine wave with unknown amplitude and frequency and added it to the noisy simulated signals. Like above, we performed a grid search to determine the optimal parameters as a function of flux (Figure 4.3C). Interestingly, the predictions for low flux values (<15 RBC/s) were greatly improved by incorporating a slow-varying component, in the form of a sine wave with frequency 1.5 Hz and amplitude of 0.3. But predictions for higher flux values were improved by a much lesser extent. Based on these findings, we again modeled RBC passages as Gaussian pulses with height, width and inter-peak time using the uniform distribution as well as the distributions found above. To secure a sufficient number of training samples of rare instances, such as low flux capillaries with high RBC speed, we sampled parameters from uniform distributions describing the mean height, width, and inter-peak time. For example, for a given flux, we sampled from a uniform distribution describing mean peak height. Now given this mean peak height, we sampled from the experimentally-determined beta distribution to obtain the peak heights of all peaks within the time-trace (Figure 4.2E). We followed a similar procedure for peak widths and inter-peak times, which have variability within each time-trace as described by gamma distributions. We found these parameters to be independent, allowing us to sample from each distribution independently. We then added noise (Eq. 4.2) with varying amplitude from 0 to 1, and sine waves with frequencies from 0 to 16 Hz and amplitudes from 0 to 0.5. We incorporated these elements by sampling from the distributions describing noise amplitude and static components as a function of flux (Figure 4.3).

4.4.4. Training of the Convolutional Neural Network on Simulated Data

Having simulated OCT time-traces of RBC passages much more realistically by adding the modeled noise and slow-varying components, we further trained the CNN of Figure 4.1 with

these realistic simulated signals, the number of which is much larger than the experimental data. We added these simulated signals to the experimental signals, producing a training sample of 10,000 training examples for each flux category, resulting in 1,20,000 total training samples.

With this data, we continued training the CNN described previously and found a modest improvement in the resulting accuracy as determined by testing the network on the experimental test data (R^2 value increased from 0.88 to 0.91, slope = 1). However, this network is more robust to different noise levels, and different RBC speeds for any given flux value, as shown in Figure 4.4. The network trained solely on experimental data systematically overestimates fluxes of simulated time-traces with smaller peaks widths, which is correlated with higher RBC speed (Figure 4.4A), even when the RBC speed is within a physiological range. Similarly, this CNN underestimates flux from time-traces with larger peak widths than average (Figure 4.4B). Simulating time-traces allows for more balanced training data, which serves to eliminate this bias.

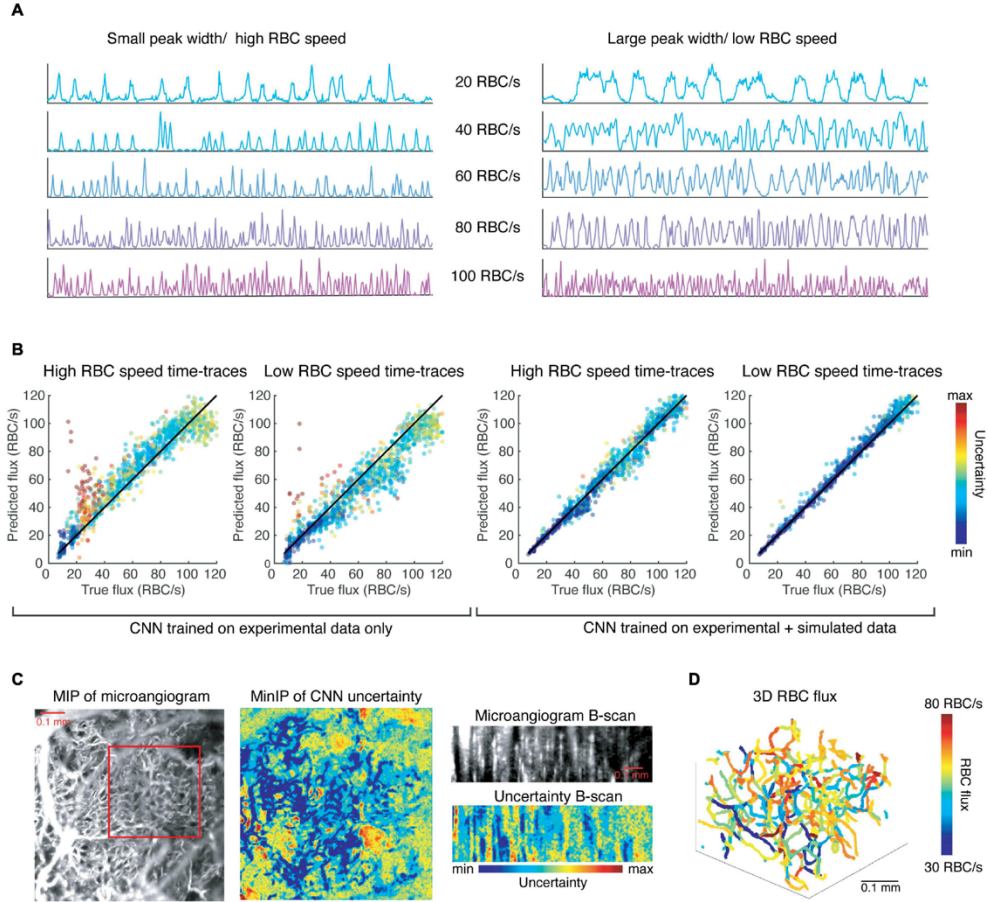


Figure 4.4. Convolutional neural network trained on simulated data is more robust to different RBC speeds (peak widths) than the CNN trained only on experimental data and can be utilized to extract RBC flux in a network of capillaries. **(A)** Simulated time-traces with the smallest (left) and largest (right) mean peak widths that were observed experimentally. **(B)** Results of the CNNs trained on experimental data and simulated data, respectively, with the input are time-traces like those shown in **(A)**. **(C)** The CNN predicts flux values with low uncertainty for voxels corresponding to capillaries. **(D)** The 3D RBC flux of the capillaries shown in the red square in **(C)**.

4.4.5. Red Blood Cell Flux of Capillary Network

We applied our CNN to 4D data (x, y, z, t) of the murine cortical vasculature to estimate RBC flux in an interconnected network of capillaries, which was unseen to the CNN during training. The data was collected over a FOV of $0.768 \text{ mm} \times 0.768 \text{ mm} \times 0.25 \text{ mm}$ (x, y, z), and for a duration of 716 ms with temporal resolution of 1.4 ms. As a sign of confidence in our method, we observed that voxels corresponding to capillaries were associated with lower uncertainty as

predicted by the CNN (Figure 4.4C). We applied our previously-developed toolbox for the segmentation of OCT angiograms to obtain the centerlines of each vessel [23]. We then applied the CNN to the time-trace of each of the voxels within the centerline of a vessel and averaged the predicted flux values for the vessel while weighting the predictions by their associated uncertainties. Repeating this prediction and averaging for every vessel in a capillary network produced a map of RBC fluxes (Figure 4.4D). While Marchand et al. used a masking technique to identify the best voxel choice, we recentered the vessel skeleton using the algorithm described in Stefan and Lee [23] to align the centerline with the voxels of lowest uncertainty. While larger datasets than the one presented here may be more uncommon (due to the large size of several terabytes of raw data acquired during imaging), the algorithm can easily be applied to such datasets. This is due to the fact that only a small fraction of voxels corresponding to vessels are selected for further processing by the CNN. This results in a running time on the order of a few minutes.

4.5. Discussion

It is useful to obtain high-throughput estimations of RBC flux, as it allows for the study of capillary network dynamics with greatly improved statistical power. OCT has been shown to be a promising tool for this purpose, considering that OCT can detect individual RBC passages due to the transient increases in intensity as the RBC passes through the imaging voxel. However, it is still unclear on how to identify these RBC passages from OCT time-traces. Basic peak-finding algorithms were an obvious starting point but have recently been shown to systematically underestimate high flux values.

By investigating time-traces obtained using OCT and TPEF simultaneously, we have uncovered the distributions governing the height, width and inter-peak time of those intensity peaks

representing the passage of RBCs. Our findings reveal that time-traces of high flux capillaries tend to have lower mean peak height, which explains why the traditional peak-counting methods underestimate RBC flux for these capillaries. Our findings also indicate that time-traces of high flux capillaries tend to have greater noise, which in turn may impact peak detection and peak width measurement. Adding noise increased the peak width by up to 17%, which may contribute to error when estimating RBC speed. We also found that the incorporation of slowly-varying components was critical to the accurate prediction of RBC flux, in particular for low flux values, suggesting that this may be a common component of time-traces of low-flux capillaries. These insights may serve to form a foundation for how researchers may simulate OCT time-traces and also some of the challenges that need to be overcome. Subsequent studies may also study the effects of multiple-scattering, lateral resolution, and the orientation of the capillary (specifically, nearly-vertically oriented capillaries). Furthermore, we believe this work extends to other mammals considering that the variation of RBC size is small [24], capillaries are of similar diameter [25], and RBC velocity appears to be independent of body mass [26].

We utilized these findings and empirical distributions to simulate OCT time-traces for a wide range of RBC fluxes and speeds, as well as noise amplitudes. This novel simulation strategy which better mimics real OCT time-traces has enabled us to develop a more balanced training set than the one acquired experimentally as well as thousands of more training examples, thereby improving the robustness of the network. We make the code freely available for researchers to implement our trained CNN directly to their data without any user parametrization, as well as code for simulating OCT time-traces and training their own networks with any input size.

4.6. Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Code and trained neural networks are available at this link:

<https://github.com/sstefan01/RBCflux>.

4.7. Ethics Statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Brown University.

4.8. Author Contributions

SS performed the analyses, acquired experimental data, and wrote the manuscript. AK performed animal surgeries. PM and FL acquired the simultaneous TPEF and OCT data. JL consulted the results and supervised the project. All authors revised the manuscript and approved the submitted version.

4.9. Funding

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4.10. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5. Near-lifespan longitudinal tracking of microvascular dynamics and structure

This manuscript is in review.

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5.1. Abstract

In age-related neurodegenerative diseases, pathology often develops slowly across the lifespan. As one example, in diseases such as Alzheimer's, vascular decline is believed to onset decades ahead of symptomology: Mechanistic understanding of such changes is key to developing early treatments that could change the trajectory of disease outcomes. However, a constellation of challenges inherent in current microscopic methods make such longitudinal tracking highly arduous. Here, we describe a suite of robust and precise methods for measuring vascular dynamics and anatomy for over seven months in the same field of view. This unique approach is enabled by advances in optical coherence tomography (OCT): We achieved long-duration high-quality imaging by developing, adopting, and integrating multiple OCT techniques and image processing algorithms, including deep learning. These integrated methods enabled us to simultaneously monitor 24 distinct vascular properties spanning morphology, topology, and function of the microvasculature across all scales: large pial vessels, penetrating vessels, and capillaries. We show the efficacy of this approach in normal mice, and differential onset of vascular changes in mice that model Alzheimer's Diseases mechanisms. This new approach will allow comprehensive and longitudinal study of a broad range of progressive vascular diseases, and normal aging, in key model systems.

5.2. Introduction

There is an increasing need for methods to longitudinally track microvascular alterations in the aging brain. As one example, in Alzheimer's disease, vascular decline is thought to emerge up to decades before the onset of cognitive symptoms and is widely viewed as one potential contributor to symptomology [1]. Similarly, changes in vascular structure and dynamics are a signature of several other progressive neurodegenerative diseases, including Parkinson's [2,3], Huntington's [4], and multiple sclerosis [5,6], suggesting a direct role in disease progression. Deriving effective early treatments for these conditions, and more generally better understanding their etiology, likely requires a better understanding of these evolving vascular changes.

However, studying the role of vascular factors in age-related neurodegenerative diseases has been challenging because of difficulties in longitudinally tracking their development. To date, multi-endpoint terminal approaches have been used for determining such vascular alterations at different ages [7-10]. While effective in some respects, these approaches are analytically less efficient, vulnerable to subject-specific random effects, and require an increasing number of animals when involving more measurement time points (Supplementary Fig. 5.1). Fluorescence multi-photon microscopy has revolutionized the *in vivo* visualization of neocortical vascular structure and function (flow) [11], but has not met the need for long-term, longitudinally repeated imaging. Bleaching and phototoxicity [12,13] and the instability of genetically-encoded indicators limit the use of fluorescent methods for stable repeated measurements across months [14-17].

Optical coherence tomography (OCT) is a label-free emerging technology that provides several unique imaging capabilities without the aid of fluorescence [18-20]. Because of its sensitivity to moving red blood cells (RBCs), OCT can rapidly produce microangiograms [21], quantitatively

measure blood flow of individual vessels [22], and even detect passage of individual RBCs in capillary vessels [23], all in the label-free manner. These capabilities suggest OCT can provide a unique solution to the longitudinal aging problem. However, it is unclear for how long OCT methods can repeatedly and robustly image the same cortical microvasculature, and the impact of aging on this imaging. More importantly, studies using fluorescence microscopy or OCT have, to date, only measured a few vascular properties at a time [11,14,15], making it difficult to investigate how degeneration in individual microvascular structural and functional properties distinctly correlate with and/or contribute to etiology of disease. A further constraint on using any method, including OCT, for such imaging is the intensive data integration and management challenges created by longitudinal acquisition. Ideally, such image sets should be processed in an automated, unbiased manner; particularly given their size, it poses another technical challenge to be addressed. As one example, the longitudinal experiment presented in this paper produced more than 50 terabytes of raw data, whose analysis would have been unrealistic without automation.

To meet the methodological need and address the related challenges, we present a set of integrated methods for tracking and analyzing diverse properties of the morphology, topology, and function of the cortical microvasculature. The approach invented for this purpose and detailed here provides simultaneous measurement of 24 vascular properties across all scales, ranging from large pial vessels to penetrating arterioles and venules to capillaries. For this comprehensive set of properties, a variety of techniques were adopted, developed, and integrated, including OCT microangiography [21], Doppler OCT [22-24], red blood cell (RBC) passage measurements [23], and several deep-learning toolkits [25] (Supplementary Text 5.1). This integrated set of imaging techniques and image processing algorithms has enabled near-lifespan longitudinal tracking of the vascular features in the aging brain. The present paper demonstrates this novel methodological capability in 3xTg model mice of Alzheimer's disease

(AD) with wild-type (WT) controls, by quantifying a process of cerebral microvascular degeneration over the course of seven months. When applied to broad age-related neurodegenerative diseases, the presented method framework is anticipated to facilitate new findings on the roles of microvascular factors in etiology and pathophysiology of disease.

5.3. Results

5.3.1. Structural and functional properties of pial and penetrating vessels

We repeated microscopic brain imaging sessions every four weeks through a chronic cranial window under anesthesia (7 AD and 6 WT mice, see Methods for details). Figure 5.1 displays an example set of OCT angiograms obtained from the same animal cortex across seven months. It demonstrates that the label-free, *in vivo* microscopy technique is stable enough for longitudinally imaging the cortical microvasculature for a long period of such duration. To quantitatively track diameter changes over the same set of pial vessels from the acquired images, we registered the images for each animal (Fig. 5.1a), selected a set of the same vessels across ages (Fig. 5.1b), and then measured the diameter of each vessel by fitting its cross-sectional profile to a Gaussian function (Fig. 5.1c). This allowed us to track the same set of 107 pial vessels over seven months (gray lines in Fig. 5.1d). These measures were analyzed by linear mixed-effects (LME) fitting to either a linear or nonlinear (sigmoidal) model, where each value was normalized by the group baseline to focus on relative changes with aging and how the changes differed between the AD and WT groups (Methods). LME analysis revealed that in AD, average pial vessel diameter decreased with age at a rate of 1.3% per month (95% confidence interval [CI], -2.4 to -0.2), but this rate was not significantly different from WT mice (-1.1% per month; 95% CI, -2.2 to 0.0; $p=0.78$ between AD and WT; Fig. 5.1e).

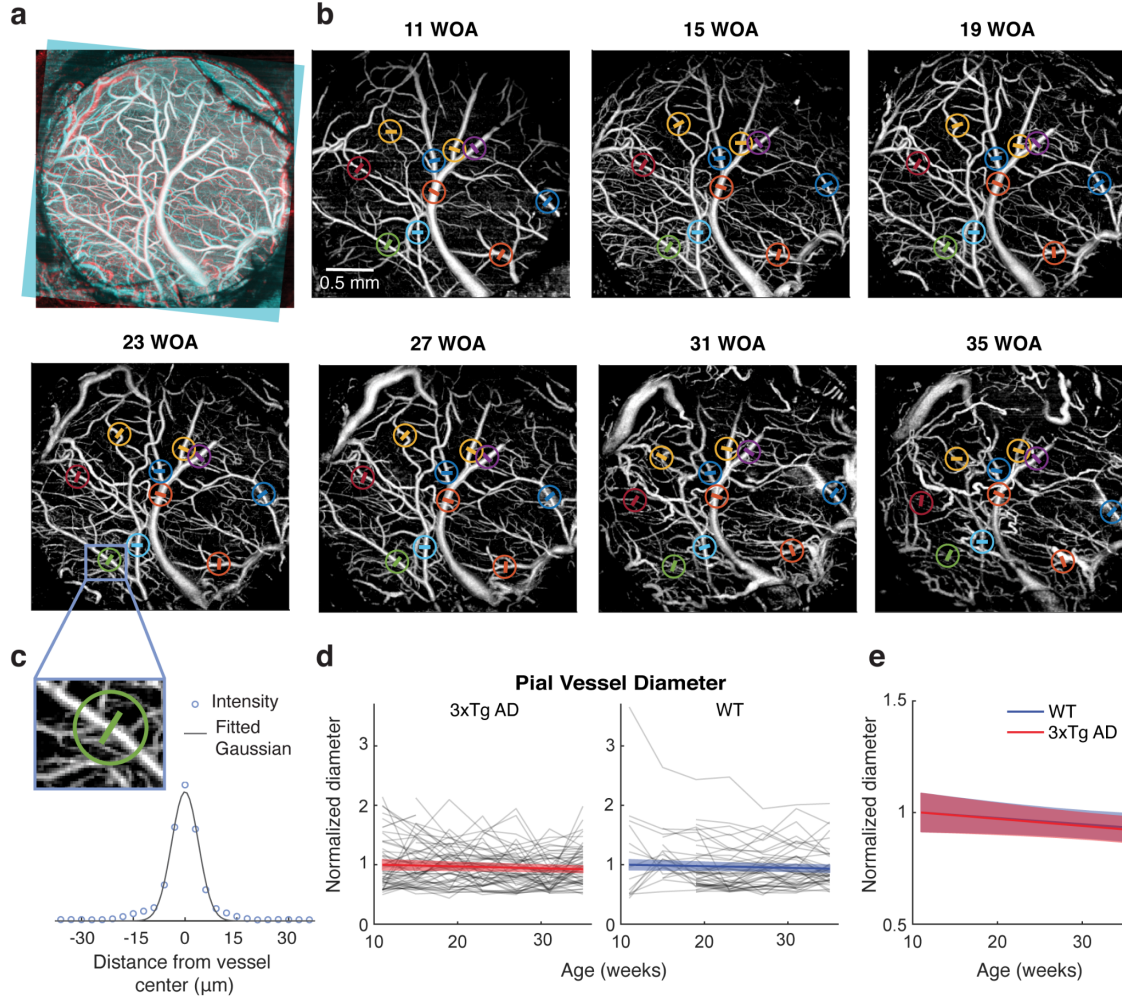


Figure 5.1. Longitudinal imaging and diameter measurement of pial vessels. **a)** An example of registration of an angiogram (cyan) to a reference angiogram (red) with overlapping regions shown in white. **b)** An example of selecting the same vessels across time points. The line indicates the automatically detected line orthogonal to the orientation of a selected vessel, along which the cross-sectional intensity profile was extracted for diameter measurement. **c)** An example of the cross-sectional profile and its fitting to a Gaussian function to measure the diameter as the full width at half maximum. **d)** Time courses of the normalized vessel diameters (gray) and their LME fits (color). **e)** The LME fits shown together. Angiograms in a) and b) are shown as maximum intensity projection (MIP). Shaded areas indicate the 95% confidence intervals.

Every imaging session included acquisition of Doppler OCT images as well. We used these images to track changes in the diameter and blood flow of individual penetrating vessels (Fig. 5.2). The diameter of each vessel was measured by fitting its *en-face* cross-section to a 2D Gaussian function, while the blood flow was measured by the area-integral method [22] after

noise reduction [24]. This enabled us to track the same set of 151 penetrating vessels for seven months (gray lines in Figs. 5.2c and 5.2f).

Arteriolar diameter showed sigmoidal decreases in both AD and WT but faster in AD, with the rates of change defined from the sigmoid fits (Methods) being -13% per month in AD (95% CI, -18 to -9) and -7% per month in WT (95% CI, -11 to -3; $p=0.008$; Fig. 5.2c). In turn, their fractional changes became significantly different between AD and WT at 18 WOA ($p<0.05$, Fig. 5.2d), which is termed as the age of significance (AOS) hereafter. Second, the venular diameter decreased in AD with the rate of change of -13% per month (95% CI, -18 to -8), significantly different from WT (1.3% per month; 95% CI, 0.9 to 1.7; $p<0.001$), leading to the AOS of 12 WOA (Fig. 5.2e). Third, the arteriolar flow decreased in both AD and WT but faster in AD (-13% versus -10% per month, $p<0.001$; AOS, 21 WOA; Fig. 5.2g). Finally, the venular flow decreased in AD only (-16% per month; 95% CI, -25 to -8; AOS, 18 WOA; Figs 5.2f and 5.2h). It is interesting to see the AOS be earlier for the structural degenerations than their flow counterparts (see Discussion for detailed interpretation).

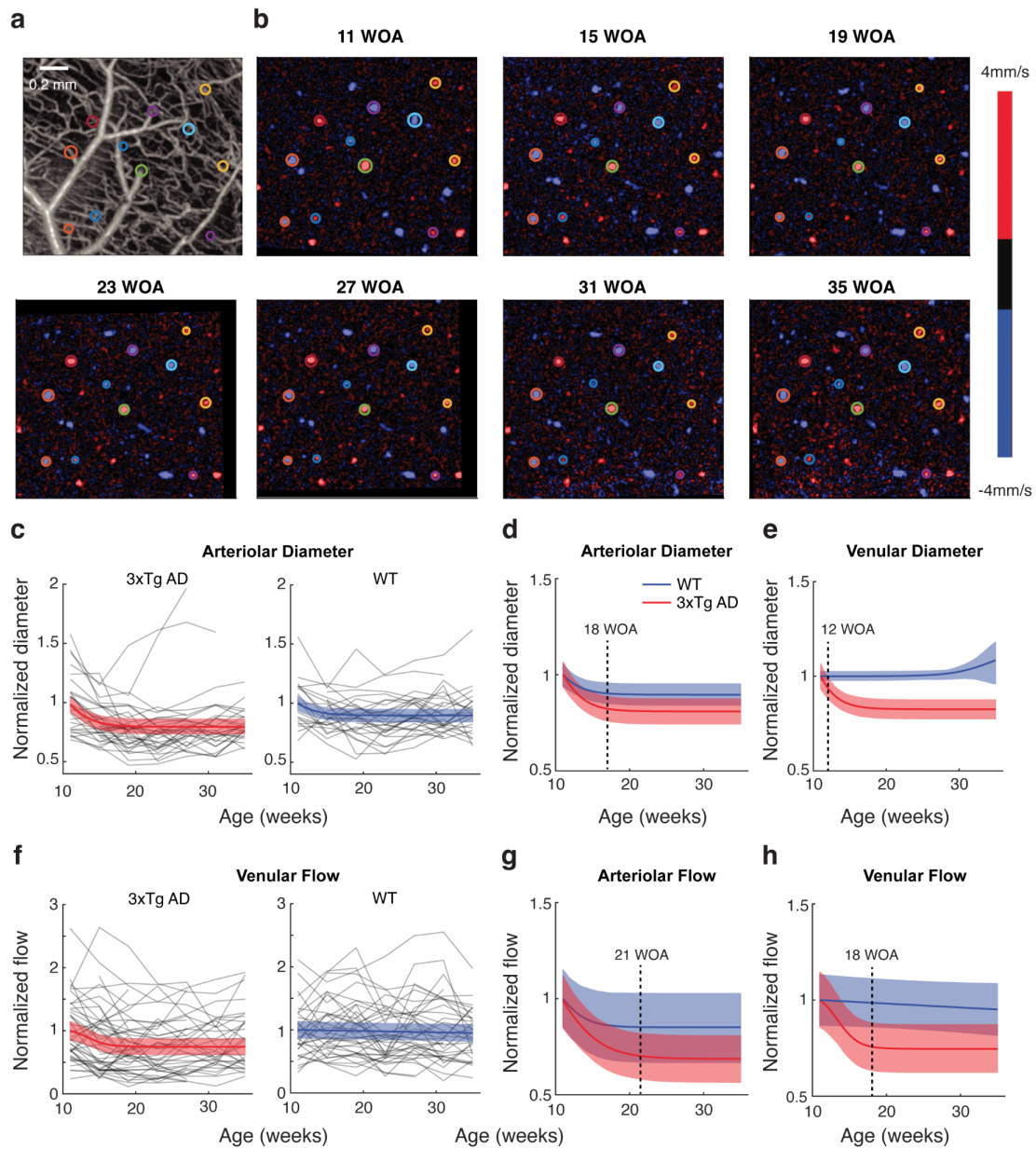


Figure 5.2. Longitudinal monitoring of blood flow and diameter in penetrating arterioles and venules. **a)** An example of angiogram MIP with overlaid circles denoting the positions of the penetrating vessels tracked in **b)**. **b)** An example set of en face slices of the Doppler velocity maps acquired from the same animal for seven months. Eleven of the same vessels were selected in this animal (circled). **c)** Time courses of arteriolar diameter of individual vessels (gray) and their LME fits (color). **d)** The LME fits of arteriolar diameter shown together. **e)** LME fits of venular diameter. **f)** Time courses of venular flow of individual vessels (gray) and their LME fits (color). **g)** LME fits of arteriolar flow. **h)** The LME fits of venular flow. Shaded areas show 95% CI, and dotted lines indicate AOS. See Supplementary Fig. 5.2 for individual vessel traces of venular diameter and arteriolar flow.

5.3.2. Structural and functional properties of capillary vessel network

To longitudinally track alterations in smaller vessels like capillaries, every imaging session also included acquisition of another OCT angiography dataset with a higher spatial resolution (3 μm) and an optical focus on the cortical capillary bed (Methods). To extract as much information as possible from these microangiography images, we first converted grayscale microangiograms into a graph of the capillary vessel network, using our deep learning-based toolbox for enhancement, segmentation and vectorization of vessels [25] (Fig. 5.3a). From these network graphs and the original grayscale images, our methods enabled measurements of numerous angio-architectural properties, including morphological properties like the length, diameter, and tortuosity of each vessel; and topological properties such as the branching order, betweenness, closeness, and shortest cycle of each vessel. Betweenness, closeness, and the shortest cycle are used in graph theory for elucidating how nodes are connected within a network (see Supplementary Fig. 5.4 for conceptual illustration and their implication when applied to a vascular network). While these properties were measured for each capillary segment (108,964 segments in total across 13 animals and seven ages of measurement), we calculated the mean and heterogeneity of every property per animal per age and then tracked them as a function of age, where the heterogeneity was quantified by the coefficient of variation (COV; the standard deviation divided by the mean). Other related measures include capillary number density, capillary length density, and fractal dimension (see Table 5.1 for a full list).

Table 5.1. Summary of statistical results of 24 vascular properties. The two numbers in the parenthesis present 95% CI. See Methods for how the rate of changes was defined when the fractional change of a property was better fit with the sigmoid function. Shown in bold are those that showed significant differences between AD and WT ($p < 0.05$) until the latest age of measurement (35 WOA).

Property	Rate of change with age in AD (%/month)	Rate of change with age in WT (%/month)	P-value of difference in rates of change between AD and WT	The age of significance (weeks)
Pial vessel diameter	-1.3 (-2.4 to -0.2)	-1.1 (-2.2 to 0.0)	0.78	
Arteriolar diameter	-13.2 (-17.5 to -8.7)	-7.2 (-11.4 to -2.8)	0.008	18
Venular diameter	-13.1 (-18.0 to -7.8)	1.3 (0.9 to 1.7)	$p < 0.001$	12
Arteriolar flow	-12.9 (-18.7 to -7.3)	-9.6 (-19.4 to -0.6)	$p < 0.001$	21
Venular flow	-16.3 (-24.9 to -7.9)	-0.8 (-2.9 to 1.3)	0.009	18
Capillary length	-1.5 (-2.6 to -0.3)	0.2 (-1.0 to 1.3)	0.005	25
Capillary length COV	-0.2 (-1.0 to 0.7)	0.1 (-0.9 to 1.1)	0.56	
Capillary diameter	0.2 (-0.4 to 0.9)	0.0 (-0.8 to 0.9)	0.63	
Capillary diameter COV	0.3 (-0.7 to 1.3)	0.5 (-0.3 to 1.3)	0.60	
Capillary tortuosity	2.2 (1.3 to 3.1)	0.7 (-0.5 to 1.9)	0.015	> 35
Capillary tortuosity COV	1.0 (-0.1 to 2.1)	3.4 (-3.5 to 10.4)	0.60	
Branching order	0.9 (-0.6 to 2.4)	1.4 (0.3 to 2.5)	0.22	
Branching order COV	1.3 (0.4 to 2.3)	1.0 (0.2 to 1.9)	0.49	

Betweenness	3.9 (1.3 to 5.8)	-0.7 (-2.8 to 1.5)	p<0.001	21
Betweenness COV	-1.7 (-3.2 to -0.2)	-0.9 (2.2 to 0.4)	0.21	
Closeness	-1.6 (-3.8 to 0.6)	-1.1 (-3.2 to 1.0)	0.67	
Closeness COV	-3.0 (-7.9 to -2.0)	3.6 (0.9 to 6.4)	p<0.001	18
Shortest cycle	1.9 (0.3 to 3.4)	-0.4 (-1.7 to 0.8)	p<0.001	20
Shortest cycle COV	-0.7 (-2.2 to 0.7)	-1.3 (2.6 to -0.0)	0.37	
Capillary number density	0 (-0.9 to 1.0)	0.0 (0.0 to 0.0)	0.23	
Capillary length density	0.6 (-0.6 to 1.9)	0.8 (-0.7 to 2.4)	0.79	
Fractal dimension	0.1 (-0.4 to 0.3)	-0.1 (-0.5 to 0.2)	0.66	
RBC flux	6.0 (3.0 to 9.0)	2.1 (0.9 to 3.2)	p<0.001	22
RBC flux COV	-6.7 (-11.4 to -2.1)	-3.0 (-4.7 to -1.4)	p<0.001	23

Prior to analyzing how the properties vary with age, we compared our measures to those reported in the literature when available. The distributions of capillary lengths, diameters, branching orders, and tortuosities obtained from our WT group (Fig. 5.3b) looked similar in shape and mean value to those previously reported from WT mice [26-29]. We also observed agreement with the literature in the capillary length density and the shortest cycle (Supplementary Tables 5.1 and 5.2).

LME analysis revealed several differences emerging between AD and WT. Mean capillary length steadily decreased in AD mice only (-1.5% per month; 95% CI, -2.6 to -0.3; AOS, 25

WOA; Figs. 5.3c and 5.3d). Mean capillary tortuosity also significantly increased in AD only (2.2% per month; 95% CI, 1.3 to 3.1), but its fractional changes did not become significantly different between AD and WT until the end of measurement (35 WOA). Regarding network topology, the shortest cycle increased in AD only (1.9% per month; 95% CI, 0.3 to 3.4; AOS, 20 WOA; Fig. 5.3e). The betweenness also increased in AD only (3.5% per month; 95% CI, 1.3 to 5.8; AOS, 21 WOA; Fig. 5.3g). In contrast, the closeness did not change significantly with aging in both AD and WT (Supplementary Fig. 5.3), but heterogeneity (COV) decreased in AD (-3.0% per month; 95% CI, -7.9 to -2.0) whereas it increased in WT (3.6% per month; 95% CI, 0.9 to 6.4), with an AOS of 18 WOA (Fig. 5.3i).

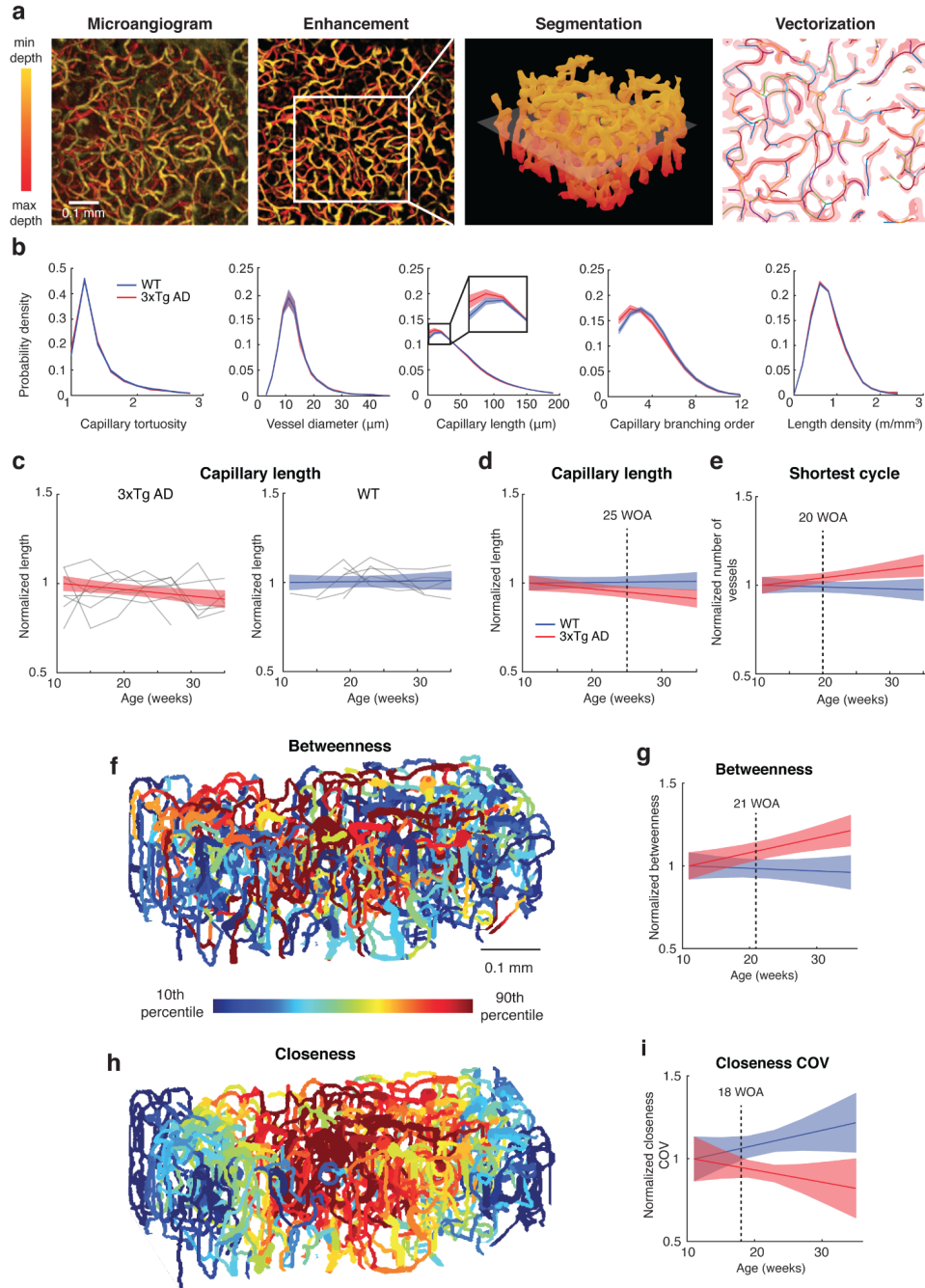


Figure 5.3. Longitudinal monitoring of microvascular morphological and topological properties. **a)** An example of the deep learning-based image processing pipeline results, from the input of microangiogram to its enhancement, vessel segmentation (all shown in MIP), and the vectorized capillary vessels. **b)** Probability density distributions of capillary tortuosity, vessel diameter, capillary length, branching order, and capillary length density. The distributions were gathered from all animals and all measured ages, for each group. **c)** Time courses of the normalized mean capillary length (gray) and their LME fits (color). **d)** The LME fits of the mean capillary length shown together. **e)** LME fits of the mean shortest cycle. **f)** An example of 3D betweenness map from an animal at one age, with each vessel colored according to its betweenness value. **g)** LME fits of betweenness. **h)** An example of 3D closeness map of the

same capillary network as (f). **i)** LME fits of closeness COV. Shaded areas show 95% CI, and dotted lines indicate AOS. See Supplementary Fig. 5.3 for all properties whose fractional changes did not become significantly different between AD and WT until the end of measurement (35 WOA).

To assess the pattern of blood flow through a large microvascular network involving hundreds to thousands capillary vessels, we previously presented a method for high-throughput measurements of red blood cell (RBC) flux based on OCT detection of RBCs passing through a voxel [23]. However, this method tends to underestimate high flux values [30] and requires user-selected thresholds. To overcome these weaknesses and improve accuracy, we developed a deep learning-based method by using two sets of RBC passage data that were simultaneously obtained with OCT and two-photon microscopy (Methods). This new method significantly outperformed the previous method (Figs. 5.4a and 5.4b, Supplementary Text 5.2).

To examine long-term changes in capillary network blood flow, every imaging session included OCT acquisition of RBC passage data, and the deep learning method was used to produce a 3D map of capillary RBC flux values (Fig. 5.4c). As a result, WT mice exhibited a gradual increase with aging (2.1% per month; 95% CI, 0.9 to 3.2; Fig. 5.4e). This increasing trend agrees with previous findings from WT rats [31] although the previous study measured the flux values only at two ages in a non-longitudinal manner. Interestingly, AD mice also showed an increase in flux with aging but at a significantly higher rate (6.0% per month; 95% CI, 3.0 to 9.0; $p < 0.001$ against WT), making the AOS to be 22 WOA (Fig. 5.4f). As can be expected from the increasing mean flux, the capillary flux heterogeneity (COV) decreased in both AD and WT but faster in AD (-6.7% versus -3.0% per month; $p < 0.001$; AOS, 23 WOA; Fig. 5.4g).

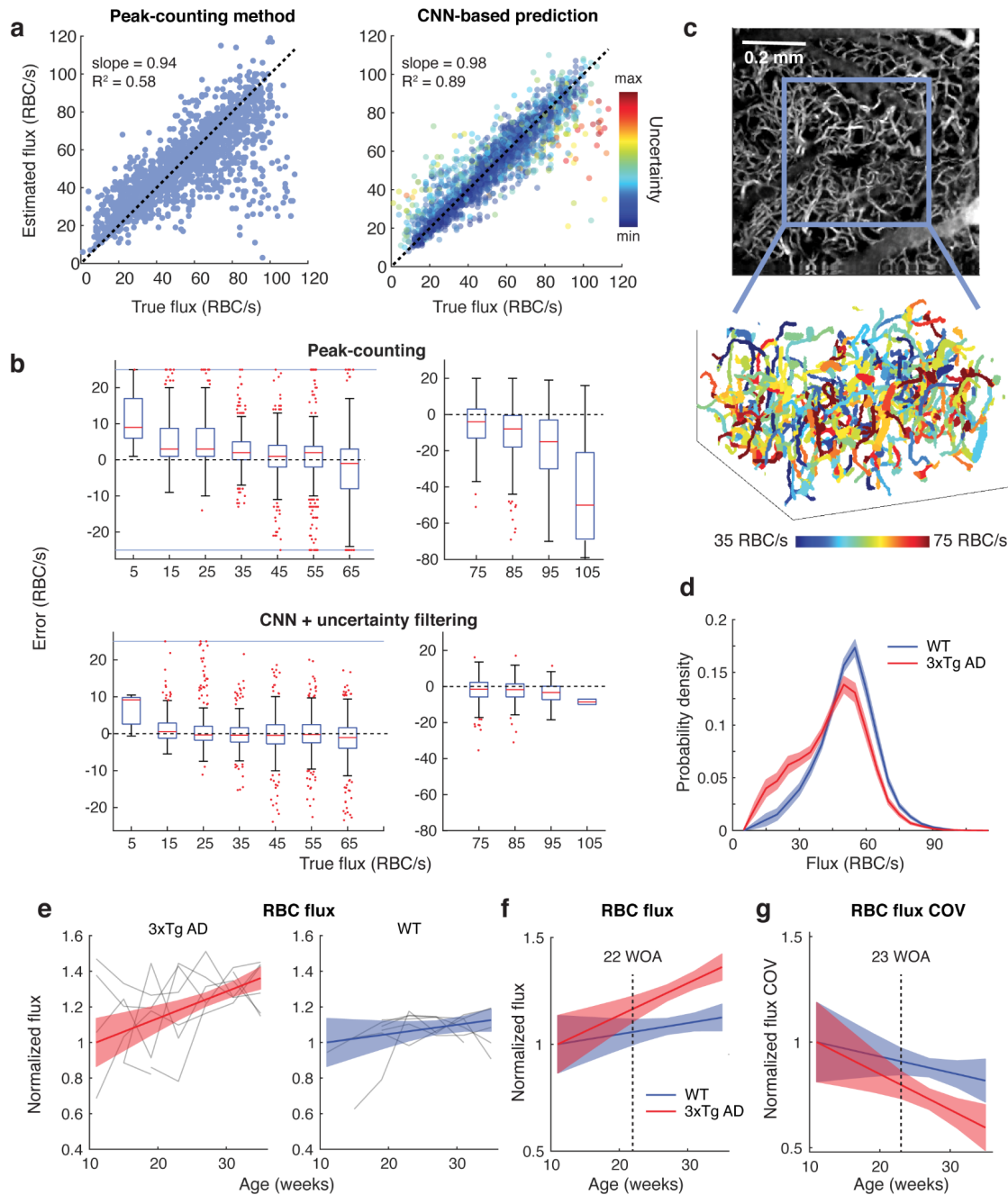


Figure 5.4. Longitudinal tracking of capillary network RBC flux. **a)** Compared to the traditional peak-counting method, the convolutional neural network (CNN)-based method produced the slope closer to 1 and higher R^2 . **b)** Distribution of errors of the two methods across different flux ranges. When the CNN-based method filtered out predictions with the lowest 20% confidence, the error became both smaller (narrower distribution) and less biased (the mean closer to 0 RBC/s) even for higher flux values. **c)** An example of microangiogram constructed from RBC-passage data (top) and the vascular graph vectorized from the microangiogram (bottom). **d)** Distribution of flux values gathered from all animals and all ages. **e)** RBC flux changes as tracked through the same animals and their LME fits. **f)** The LME fits of RBC flux shown together. **g)** LME fits of the RBC flux COV. Shaded areas show 95% CI, and dotted lines indicate AOS.

5.3.3. Temporal relationships between microvascular degenerations and cognitive impairment

To simultaneously obtain the time course of cognitive impairment, the animals also underwent novel object location (NOL) tests every month (Methods; the NOL test assesses spatial cognition and memory [32]). While WT mice showed no significant changes in the discrimination index of NOL, AD mice showed cognitive decline with the discrimination index becoming significantly lower than WT at 27 WOA (Figs. 5.5a and 5.5b), consistent with previous findings from the identical 3xTg AD model [33].

As listed in Table 1, ten out of 24 vascular properties showed significant differences in fractional changes between AD and WT during the ages of measurement (11 to 35 WOA). To illustrate the temporal relationships of these vascular degenerations to the cognitive impairment, we plotted a chronological graph as shown in Fig. 5.5c. This graph revealed three interesting relationships. First, all the observed vascular degenerations preceded the cognitive decline, up to 15 WOA early (about a seventh of the typical lifespan). Second, degenerations in arterioles and venules appeared earlier than those in capillary vessels. Third, structural changes generally preceded corresponding flow changes (arteriolar/venular diameter versus flow, and capillary betweenness/shortest cycle versus RBC flux; see Discussion for interpretation).

To reveal the interrelationships among vascular alterations, we calculated the correlation between their time courses, with age lags of 0, 4, 8 and 12 weeks (Methods). We found 25 pairs with significant correlations in AD and 18 pairs in WT, with eight pairs common to both (Fig. 5.5d). Some relationships are unsurprising, such as the positive correlation between diameter and flow changes in the penetrating vessels, which is present in both AD and WT. Some other relationships may present new findings: for example, the betweenness formed the greatest

measurement. The graph displays their rates of change in AD at their AOS (structural properties in purple, and flow properties in green). **d)** Significant correlations between age-related vascular alterations ($p < 0.05$ after Benjamini-Hochberg multi-hypotheses correction). Pairs with positive and negative correlations are shown in red and blue respectively. More opaque lines indicate higher maximum correlation, and thinner lines means that the maximum correlation appeared at longer age lags. A correlation with an age lag means that one alteration was correlated to the other one with a certain time delay.

5.4. Discussion

We have demonstrated that the integrated set of label-free in vivo imaging and image-processing methods produces an unprecedented set of data regarding age-related alterations in the morphology, topology, and function of the cortical vasculature across all scales. Such information-rich, multi-feature, longitudinal datasets allow to simultaneously investigate multiple aspects of slowly developing cerebral microvascular degeneration while affording high statistical power (Supplementary Fig. 5.1). Among other results presented here, the chronological and correlation graphs (Figs. 5.5c and 5.5d) are a representative example of what type of information can be obtained by the presented methods. Such information may provide insight into how different types of vascular alterations develop along with or independently of each other, and how the development pattern differs between physiological and pathological aging brains.

In the presented demonstration, we stopped the longitudinal imaging when the AD mice exhibited obvious differences from WT in the cognitive function test for two consecutive sessions (i.e., for eight weeks). The tracking period was approximately a third of the typical mouse lifespan. The potential tracking period could be even longer as the image quality did not significantly degrade over time in many animals (Fig. 5.1b for example). One limitation in the presented demonstration is that we conducted all imaging experiments under anesthesia; however, the presented methods are readily applicable to awake imaging as shown in our

follow-up studies. Supplementary Text 5.3 provides further discussion of other challenges and opportunities related to the presented methods. Supplementary Figure 5.7 summarizes how the various types of OCT data went through the described image-processing pipeline, and all processing software codes are publicly opened (see Code Availability).

In our results with the 3xTg model (see Supplementary Text 5.4 for argument regarding the used animal model), the earliest cerebral microvascular degeneration (CMD) was detected in the diameter of penetrating vessels (thinner in AD mice), followed by degeneration in their blood flow (lower in AD). It is interesting to see the structural degenerations precede the flow counterparts (Fig. 5.2). We speculate that once penetrating vessels start becoming pathological in structure (i.e., becoming statistically different from age-matched WT), a compensating adaptation occurs to autoregulate cerebral blood flow. However, this adaptation may not be sustained indefinitely, and eventually blood flow in penetrating vessels decreases below the normal physiological range (95% CI of age-matched WT; see Supplementary Text 5 for further argument).

On a smaller scale, the presented results revealed degenerations in both morphology (the mean capillary length) and topology (the shortest cycle, betweenness, and closeness COV) of capillary vessel networks. The mean capillary length result (becoming shorter with aging in AD only, Fig. 5.3d) is consistent with the previous finding that capillary length is shorter in AD than WT26, although the previous study did not longitudinally track the length and used a different AD mouse model. In contrast, the topological properties have been little studied in the context of aging. Interestingly, the topological changes became pathological earlier than the morphological change (Fig. 5.3). Also, both the shortest cycle and the mean betweenness increased with aging in AD, likely indicating a higher degree of susceptibility to vascular insult than WT (Supplementary Text 5.6). This higher susceptibility may be important in AD when considering

the increased rate of capillary stalling in AD models [34]. The presented results also showed degenerations in capillary blood flow (see Supplementary Text 5.7 for interpretation).

Finally, the correlation graph (Fig. 5d) revealed clear differences between AD and WT in how distinct, age-related vascular alterations correlate with each other. Of all the vascular alterations monitored, the betweenness of capillary networks formed the greatest number of correlations with other alterations in both AD and WT, but its correlation pattern differs between AD and WT. The betweenness was negatively correlated with its COV in WT, as can be expected in normal aging, and was not coupled with the mean capillary length (theoretically independent properties). In AD, however, the betweenness was uncorrelated with its COV and became coupled with the capillary length (another pathological property). Considering that the betweenness exhibited clear pathological alterations in AD only, the earliest among the topological properties (Figs. 5.3g and 5.5c), this correlation result sheds light on the betweenness of the capillary network as an important component of CMD in neurodegenerative diseases. This spotlight on the relatively novel topological property in aging research, now being longitudinally measurable in vivo by the presented methods, encourages further studies on how other vascular/non-vascular factors interact with capillary network betweenness and how the interaction in turn makes the vascular and other related systems pathological.

Future findings enabled by the presented methods in diverse AD models, when combined with follow up studies at cellular/molecular level, may determine whether CMD is etiological in AD pathogenesis, one of the long-lasting questions in AD research³⁵. Such findings can suggest updating the AT(N) framework to AT(VN) for the Alzheimer's disease continuum³⁶, and more importantly, will lead to the development of therapeutic targets as well as biomarkers for preemptive early diagnosis. When widely applied beyond AD, the presented methods are expected to provide new insight into progressive development of vascular etiology and

pathophysiology, or a novel means to monitor long-term vascular responses to therapeutic intervention, in a range of age-related neurodegenerative diseases.

5.5. Methods

5.5.1. Animal preparation

Anesthesia and Perioperative Monitoring. Male wild-type (WT) mice (n = 10) and male 3xTg AD (n = 10) underwent craniotomy for window placement [37] and in vivo OCT imaging under inhaled anesthesia. Of these mice, 4 WT mice and 3 AD mice died during the experiment or were excluded due to degrading image quality. All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Brown University and Rhode Island Hospital. Experiments were conducted according to the guidelines and policies of the office of laboratory animal welfare and public health service, National Institutes of Health. Mice underwent induction of anesthesia with 3% isoflurane in oxygen and were then maintained at 2% isoflurane until recovery in 100% oxygen. For both the initial surgery and subsequent imaging sessions, the duration of anesthesia from induction to recovery was approximately 2 hours. Oxygen saturation, pulse rate, and temperature were continuously monitored with a pulse oximeter and rectal probe for the entire surgical procedure and imaging sessions. Body temperature was maintained at 37 °C and the pulse remained within the normal range of 250-350 bpm.

Craniotomy. After induction of anesthesia, the scalp overlying the parietal bones was shaved and then sterilized using alcohol and iodine scrub. A 1-cm midline incision was made on the scalp, and the skin on both sides of the midline was retracted. The pericranial tissue was stripped using needle-tip forceps. A proprietary metal frame, used to hold the head and prevent motion during microscopy, was affixed to the skull with dental cement (Parkell Inc., Long Island,

NY, USA). A 3-mm bone flap overlying the left parietal cortex was thinned with a dental burr until transparent (100- μ m thickness). The thinned bone flap was removed with fine-tip forceps while keeping the dura intact and the skull defect filled with normal saline until hemostasis was achieved. A 3mm circular glass cranial window with a 5mm circular glass cover slip was then placed into the defect. The window was then fixed in place and the annular skull defect surrounding the window was sealed using dental cement. The cement was given time to fully cure, and the mouse was then recovered in an oxygen chamber. Mice were housed in individual cages postoperatively to protect the integrity of the metal frame.

The use of an open-skull cranial window has clear strengths and weaknesses against a thinned-skull cranial window. The open-skull method in this study provides higher quality microangiography where individual capillaries are clearly visualized (Fig. 5.3a) and individual RBC passages through those capillaries are clearly captured in the time courses of the image intensity signal (Supplementary Fig. 5.5). However, the open-skull method is known to be relatively less robust long term; we began with 20 animals, but 4 animals did not survive until the end of our seven-month longitudinal experiment. Additionally, 3 animals survived and remained healthy but the imaging quality through the window degraded significantly with time, secondary to scar tissue formation underneath the window such that insufficient number of vessels were visible with imaging - these animals were not included in our analysis. We also note that installing a chronic cranial window in mice at younger than 10-12 weeks can be problematic due to incomplete skull growth prior to this age [38].

5.5.2. OCT imaging

All OCT imaging was performed in vivo. Baseline OCT imaging was obtained seven days following the surgery, and subsequently each animal underwent imaging every four weeks for seven months. After induction of anesthesia and for the duration of imaging, the mouse's head

was stabilized against motion by attaching the affixed metal frame to a proprietary platform with a heating pad and inhaled isoflurane delivery system underneath the OCT system.

All OCT measurements were collected with a commercial SD-OCT system (Thorlabs, Newton, NJ, USA). The system uses a large-bandwidth near-infrared light source with center wavelength of 1310 nm, and wavelength bandwidth of 170 nm, which leads to a high axial resolution of 3.5 μm . The system uses a high-speed 2048-pixel line-scan camera to achieve 147,000 A-scan/s. During each imaging session, we acquired angiograms of large pial vessels using a 5X objective lens, with a field of view (FOV) of 1024 x 1024 x 512 (x,y,z) corresponding to an imaging volume of about 3 x 3 x 1.8 mm. Microvessels were imaged using a 10X objective lens with a FOV of 1024 x 1024 x 512 (x,y,z) corresponding to an imaging volume of about 1.5 x 1.5 x 1.8 mm (x,y,z). We measured blood flow in penetrating vessels using Doppler OCT by repeating 8 A-scans at each (x,y) position over a FOV of 512 x 512 x 512 (x,y,z) corresponding to a region of about 1.5 x 1.5 x 1.8 mm. RBC flux data consisted of 512 repeated B-scans with a temporal resolution of 1.4 ms, producing a 4D dataset of size 256 x 256 x 512 x 512 (x,y,z,t), corresponding to an imaging volume of 0.8 x 0.8 x 1.8 mm (x,y,z) over a time period of 0.7 s.

5.5.3. Microangiography

We measured the capillary length, diameter, and tortuosity as previously described [25], the histograms of which are shown in Fig. 5.3b. However, to track tortuosity with aging, we opted instead to measure the mean curvature of the vessels, calculated by the mean derivative of the tangent along the vessel. To determine branching order, we leveraged the fact that penetrating vessels appear as dark circles (or shadows) in the structural intensity image. This allowed us to label all penetrating vessels and trace connected vessels as they branched out, thereby finding the closest distance between each capillary and a penetrating vessel along the capillary bed.

5.5.4. Capillary network properties

Closeness and betweenness aim to elucidate how vessels are connected within the network. Closeness is calculated as the reciprocal sum of the number of vessels along the shortest paths between the vessel and every other vessel in the network. Betweenness counts how many times a single vessel is traversed along the shortest path between any other two vessels within the network (Supplementary Fig. 5.4). However, these metrics depend on the size of the network, so to account for this we divided the mean values by the mean closeness or betweenness of an idealized honeycomb network with the same number of vessels. Supp. Fig. 5.4d shows the relationship between mean closeness and betweenness and number of vessels in a honeycomb network, which was used to normalize the values obtained from the experimental data. The COV was normalized in a similar manner.

Lastly, we looked at global properties of the network within the imaging volume: we calculated the capillary number density by dividing the number of capillaries by the volume, and the capillary length density by summing the lengths of all capillaries and dividing by the volume. Fractional dimension was determined using the box-counting method [39].

5.5.5. Capillary RBC flux

Data preparation. We developed the CNN-based, RBC flux-measuring method by utilizing two sets of RBC passage data that were simultaneously obtained with OCT and two-photon microscopy (TPM). We trained a 1D CNN using the OCT time traces as the training data, and the corresponding RBC flux determined from the TPM time traces as ground truth (Supplementary Fig. 5.5). We augmented this dataset by reversing the time of the time traces, and split this dataset into training, validation and testing as 70%, 20% and 10% respectively.

CNN architecture and training. We implemented the state-of-the-art architecture in time-series classification, InceptionTime40, as shown in Supplementary Fig. 5.6. We designed our CNN to provide an estimate of uncertainty in its prediction by adapting the loss function as shown in Eq. 1 to maximize the probability of a given prediction ($\hat{y}$) assuming this prediction is derived from a normal probability distribution with mean equal to G , and standard deviation, σ . G is the ground truth, and the standard deviation thus serves as an indicator of uncertainty in the predicted value. We trained our network using the Adam optimizer, with a learning rate of 0.0008 for 50 epochs, and a gradient threshold of 500 which was necessary given our adapted loss function.

$$loss = -\log \left(\frac{1}{\sigma\sqrt{2\pi}} \exp \left(-\frac{(G-\hat{y})^2}{2\sigma^2} \right) \right) \quad (5.1)$$

RBC flux estimation. To track long-term changes in the capillary network blood flow pattern, we acquired 4D data (x,y,z,t) consisting of 512 timepoints over a period of 0.72 seconds. We first constructed an angiogram from this data (Fig. 5.4c) and obtained a vascular network graph from the image as described in Fig. 5.3a. We extracted the time course from the 4D data for each centerline voxel of the graphed vessels, where RBC passage is known to cause a transient increase in intensity. We then applied the CNN to each time course along the centerline and averaged the predicted flux values along the centerline voxels for each vessel by weighting the predictions according to their associated uncertainties, thus determining the average RBC flux for each vessel (Fig. 5.4c, bottom). Finally, we determined the mean flux for each dataset (per animal per time point) by averaging the flux values over all vessels, and the COV by dividing the standard deviation by the mean.

5.5.6. Novel object location (NOL) test

Every four weeks from 11 weeks of age, each mouse was cognitively evaluated using a novel object location test that is widely used to assess spatial memory [41]. Our test used an open-

field apparatus consisting of a square arena ($40 \times 40 \times 49 \text{ cm}^3$). The mouse was habituated to an empty arena with four visually-distinct quadrants for 5 minutes, and then removed. The arena was cleaned with 70% ethanol and dried to eliminate any potential odor cues left beforehand. Then, two identical objects were placed into the northwest (NW) and northeast (NE) quadrants, while the other quadrants remained empty. The mouse explored the arena for 5 minutes while video tracked its movement. The mouse was removed, the arena was again cleaned, and both objects were placed again in the NW and southeast (SE) quadrants such that one of the objects was placed in a different location (SE). After 20 minutes, the mouse was again placed into the arena and allowed to explore the arena for 5 minutes while its movement was tracked. Differences in movement between the first and second 5-minute tracking were used to assess recognition of the novel object's location.

To determine the sign of cognitive decline, we calculated the discrimination index:

$$Z = \frac{T_{SE}}{T_{NW} + T_{SE}} \quad (5.2)$$

where T_{SE} is the amount of time spent exploring the object in the novel position, and T_{NW} is the amount of time spent exploring the object in the same position. A z-score of 0.5 or below was considered indicating that the mice did not remember one object being moved to a novel location, and thus was considered as the sign for cognitive decline [41].

5.5.7. Statistical analysis

We used the linear mixed-effects (LME) method to fit each set of measured time courses to

either a linear model or a nonlinear model following a sigmoidal curve, $y = c + \frac{L}{1 + \exp(k(b - age))}$,

and then selected the model with the lowest Akaike Information Criterion (AIC). The AIC also informed our choice of random effects based on the intercept, slope and/or time-group

interaction. We normalized each metric for AD and WT groups by the intercept as determined by the fitted model. Outliers were excluded based on Cook's distance: any value with a Cook's distance greater than 3 times the mean was considered an outlier.

The rate of change was simply defined by the slope for the linear model. For the sigmoid model, we defined the rate of change by measuring 90% of the total changes observed during the ages of measurement (11-35 WOA; from the bottom 5% to the top 5%) and then by dividing the 90% change by the age duration corresponding to the 90% change. The same was done on the 95% confidence intervals of the nonlinear fitting to obtain the confidence intervals for the nonlinear slope. When comparing the rate of change between AD and WT, for linear models, we recorded the p-value of the interaction term to determine significant differences between slopes. For nonlinear models, we determined the p-value by conducting a t-test between the estimated parameters L for each nonlinear model. We selected vascular properties for further analysis which were associated with significant p-values at an overall level of $\alpha = 0.05$ after Benjamini-Hochberg correction to control false discoveries. For these metrics, we determined the age at which AD and WT groups differed by assessing the degree of overlap between two confidence intervals. To account for multiple hypothesis testing using Bonferroni correction, we aimed to find the age at which the groups were significantly different at a rate of $\alpha = 0.05/7$, which in turn leads to the age at which the 93% confidence intervals just touch.

5.5.8. Correlation analysis

To study the interrelationships between different vascular factors and cognitive decline, we determined the correlation between each metric pair over all animals in each group, with time lags of 0, 4, 8 and 12 weeks. We calculated the corresponding p-value of each pair using a Student's t-test and corrected for multiple hypothesis testing using the Benjamini-Hochberg procedure. Significant correlations are shown by a line adjoining the two metrics (red and blue

for positive and negative correlations respectively) in Fig. 5.5d. The maximum absolute correlation is shown by the opacity of the lines, with more opaque lines representing higher values, and the thickness of the lines depicts the time lag at which the maximum absolute correlation is achieved, with thinner lines representing larger time lags.

5.6. Data availability

The raw image data is larger than 50 terabytes in size, difficult to be shared online, but all the measured property values used in statistical analysis can be found at <https://doi.org/10.6084/m9.figshare.19178885>.

5.7. Code availability

All codes for image processing and analysis were developed in MATLAB. The MATLAB codes are available for non-commercial use in GitHub (<https://github.com/optobrain/adp-1-3xtg-cortex>).

5.8. Acknowledgements

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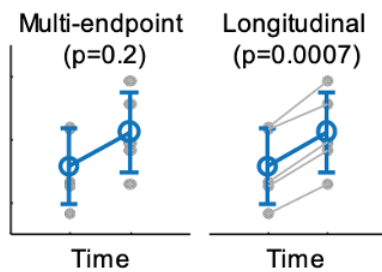
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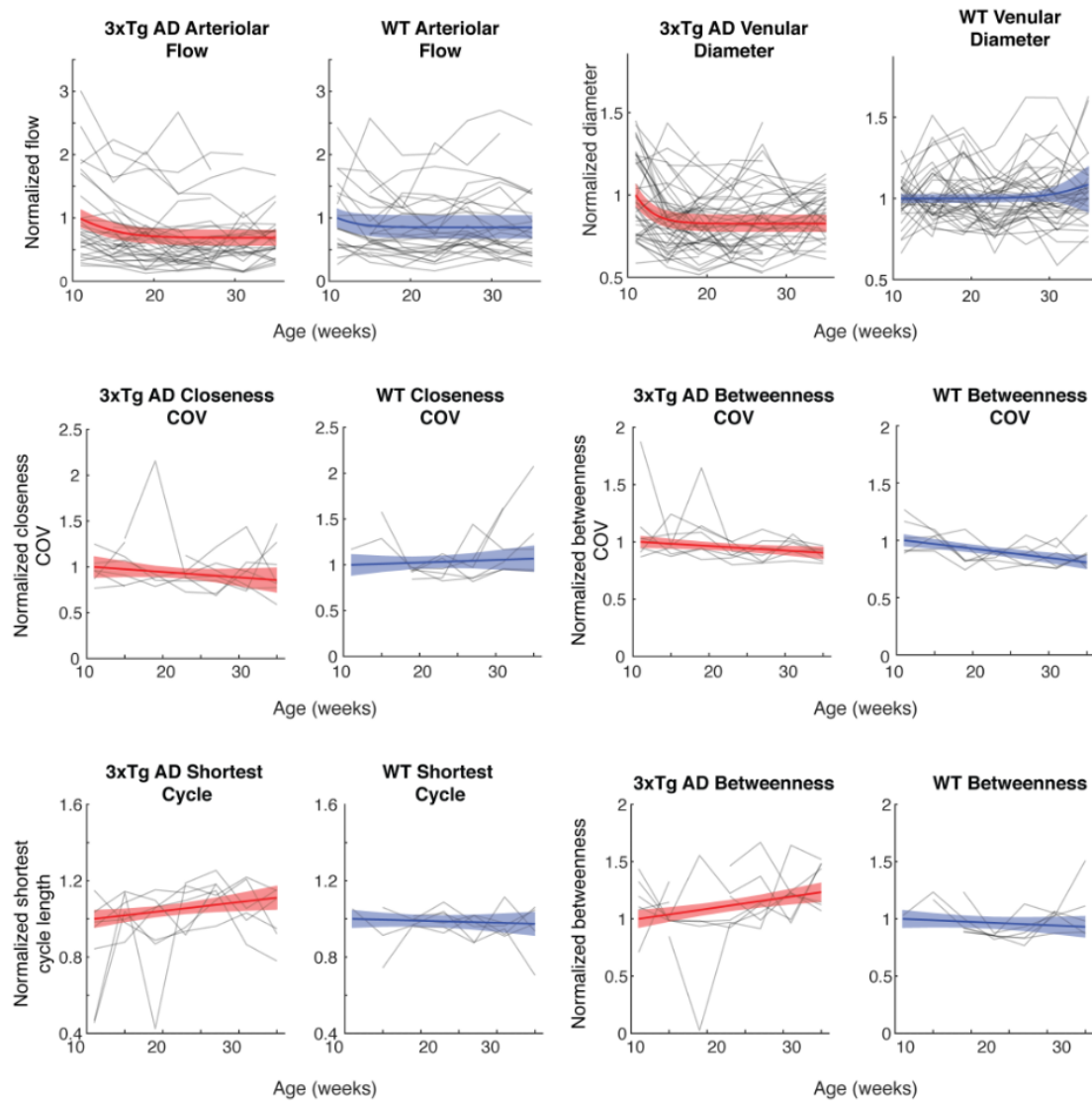
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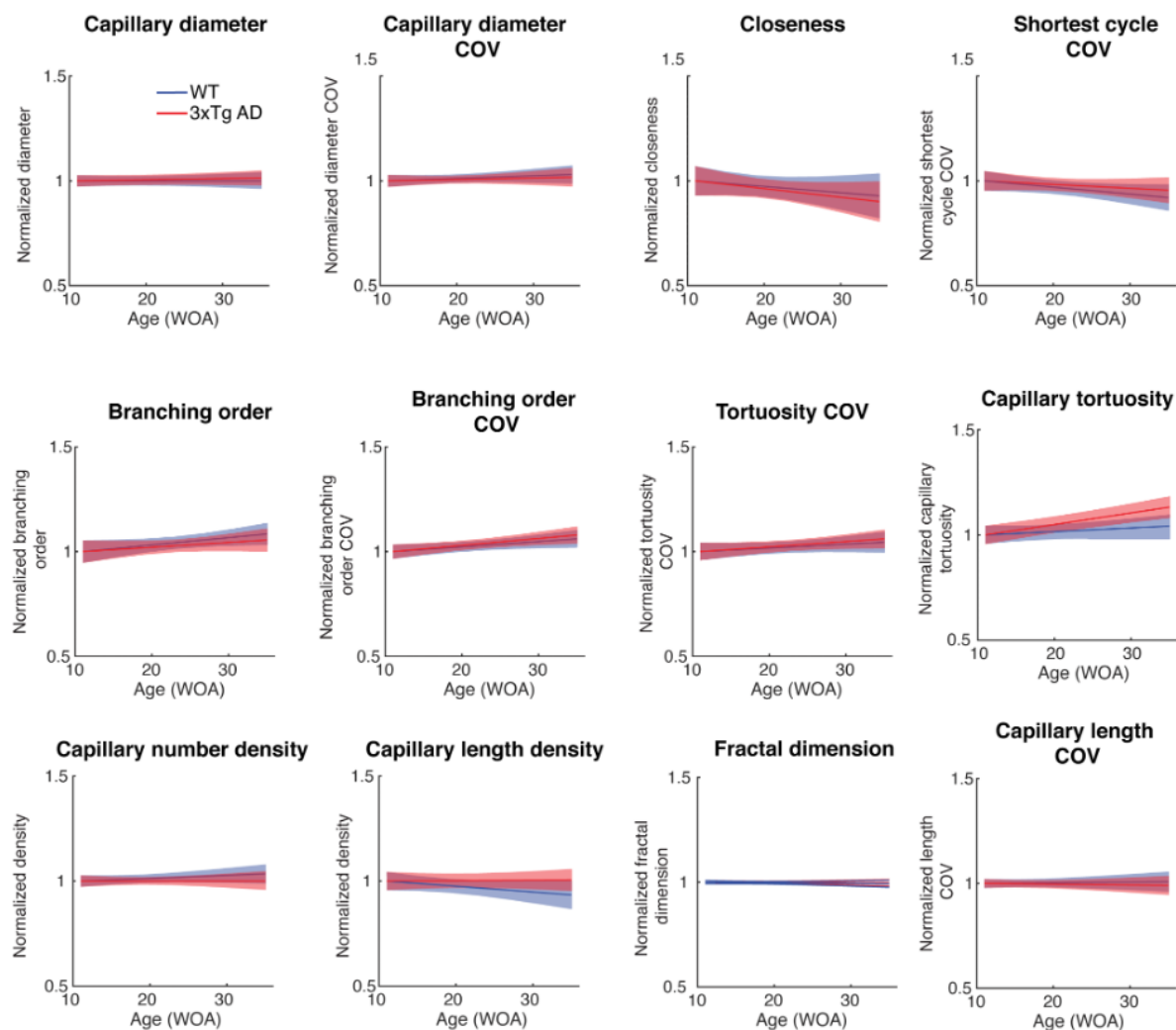
5.10. Supplementary information



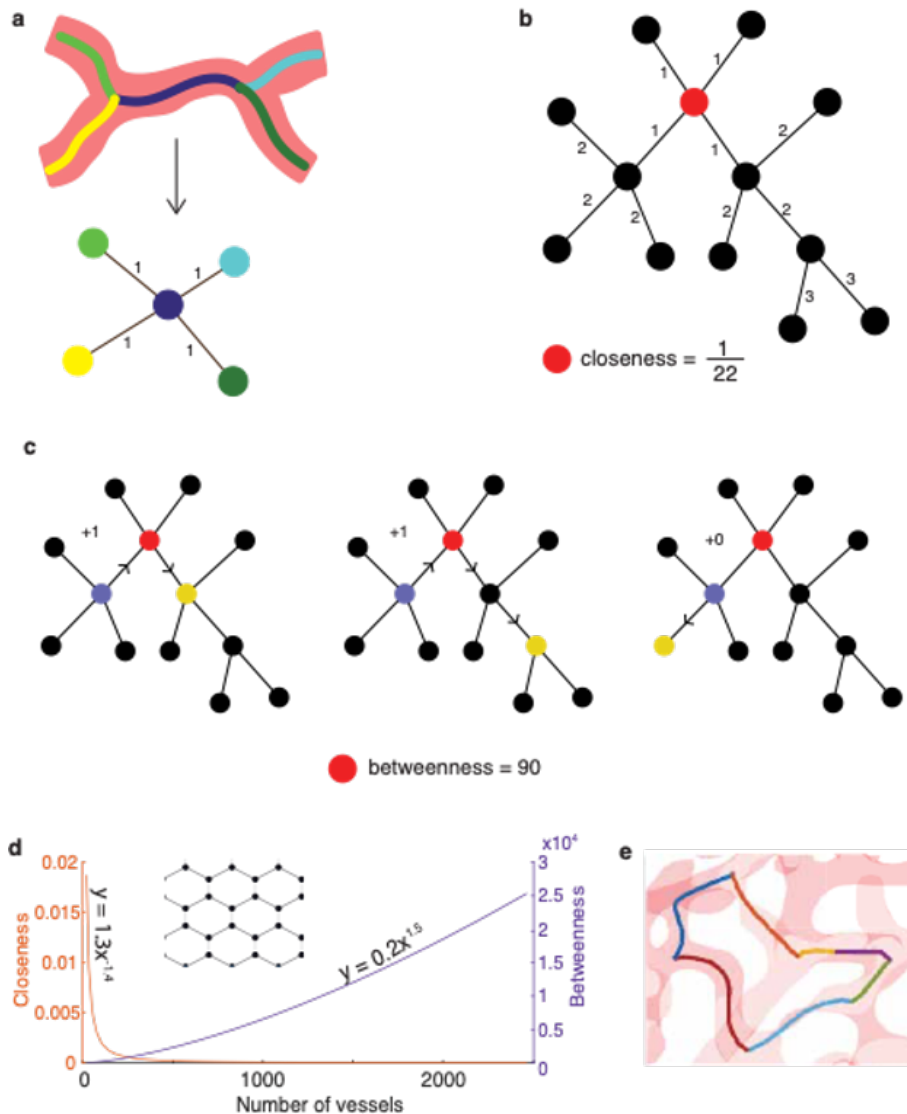
Supplementary Figure 5.1. An example of the multi-endpoint vs. longitudinal approach. This simulated data shows different p-values resulting from the identical mean and standard deviations. The illustrative data was simulated by using a simple random number generation in MATLAB.



Supplementary Figure 5.2. Time courses of vascular parameters which were significantly different between 3xTg AD and WT mice.

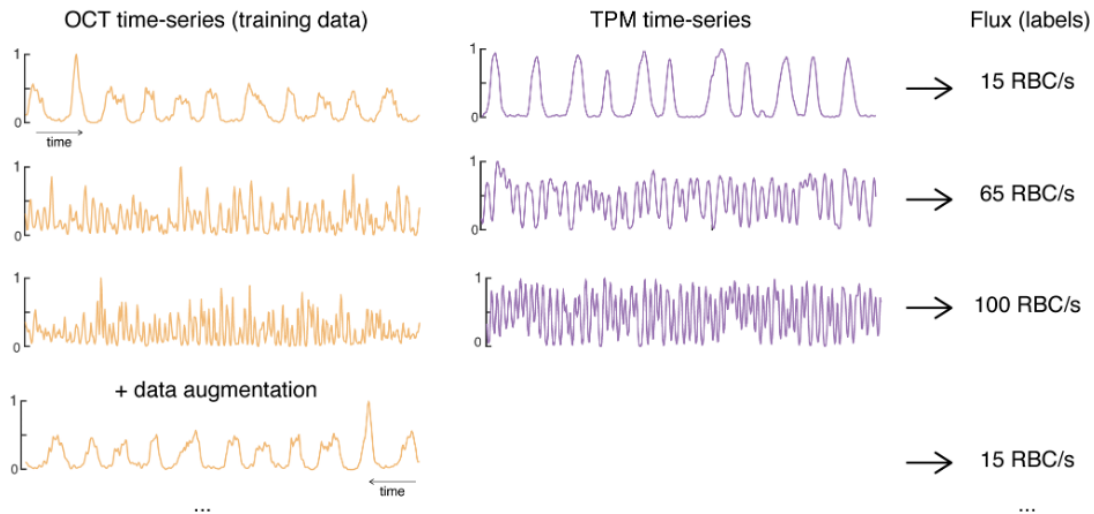


Supplementary Figure 5.3. All properties which either had statistically no difference in the rates of change between AD and WT or had statistically different rates but the fractional changes did not become statistically different between AD and WT until the end of experiment (35 WOA), except for the pial vessel diameter which was shown in Fig. 5.1.



Supplementary Figure 5.4. Topological properties of a capillary vessel network. Closeness is calculated for each vessel as the reciprocal sum of the number of vessels along the shortest paths between the vessel and every other vessel in the network. Betweenness counts how many times a single vessel is traversed along the shortest path between any two other vessels within the network. The closeness quantifies relative importance of a vessel to the network, and the betweenness indicates the extent to which the network would be vulnerable to malfunction of the vessel. The shortest cycle represents the mean number of vessels traversed in the shortest loop to return to the same vessel, serving as an indicator of overall connectedness of the vessel. **a)** For conceptual illustration, five vessels are shown, each in a different color, and their representation as nodes and edges. The path length between any two vessels which are directly connected is defined as 1. **b)** An example of how closeness is determined for the red node: the path length for all nodes from the red node are summed and inverted. **c)** Betweenness is an indication of the number of times a node is along the shortest path between any two other nodes: depicted is the scenario when the red node is along the shortest path between the purple and yellow nodes (left two diagrams) and the scenario when the red node is

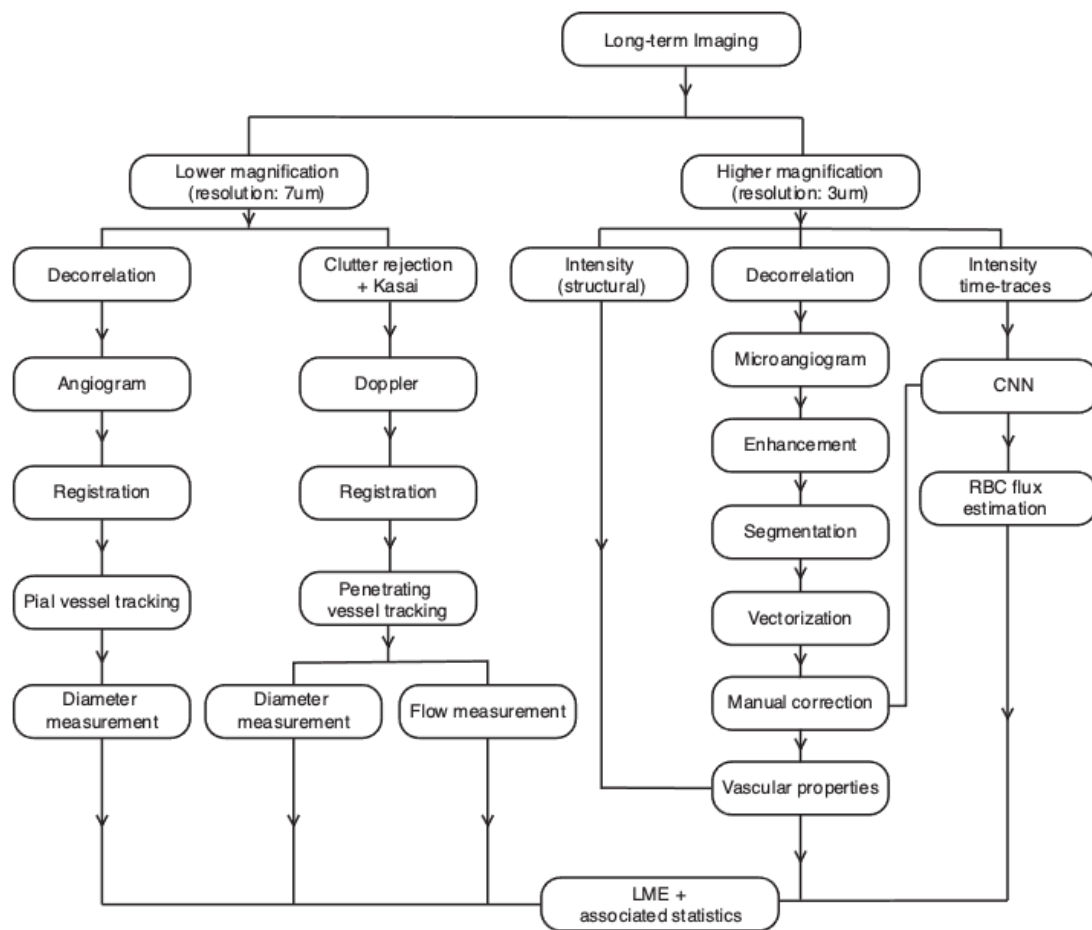
not along the shortest path (right diagram). **d)** The relationship of mean betweenness and closeness with respect to the number of vessels in a capillary network idealized as a honeycomb network. **e)** The illustration shows an example of the shortest cycle for one capillary. This is defined as the smallest number of capillaries that need to be traversed before returning to the starting point.



Supplementary Figure 5.5. Examples of time-series of RBC passages acquired simultaneously using OCT and TPM for low, average, and high flux values. The TPM time-series were used to obtain the “ground truth” for RBC flux. The OCT data was additionally augmented by flipping the time axis, thereby doubling the total training data.



Supplementary Figure 5.6. InceptionTime 1D CNN architecture for the prediction of RBC flux from OCT time series data.



Supplementary Figure 5.7. Flowchart of analysis of longitudinal imaging data.

Supplementary Table 5.1. Comparison of angioarchitecture properties available in the literature.

	This work (mean and 95% CI)	Ji et al. [1]	Kirst et al. [2]	Todorov et al. [3] (mean ± SE)	Blinder et al. [4] (median)	Schager et al. [5]
Branching order	3.7 (3 to 4)	3.4 ± 0.2 (mean ± SD)				

Capillary length (μm)	41 (33 to 48)				50	
Capillary tortuosity	1.27 (1.23 to 1.34)	1.27 ± 0.05 (mean $\pm$ SE)				~ 1.17
Shortest cycle	7.8 (5.3 to 10.3)	8.97 ± 0.53 (mean $\pm$ SE)			8	
Capillary length density (m/mm^3)	0.74 (0.67 to 0.80)	0.97 ± 0.03 (mean $\pm$ SE)	0.42	0.63 ± 0.09		~ 1.1

Supplementary Table 5.2. Comparison of angioarchitecture properties available in the literature.

	Gould et al. [6]	Haft-Javaherian et al. [7]	Ji et al. [1]	Blinder et al. [4]	Kirst et al. [2]
Branching order			Fig. 3g		Fig. 4i
Capillary length	Fig. 2b	Fig. 7	Fig. 5d	Fig. 2a	
Capillary tortuosity		Fig. 7	Fig. 3c		
Shortest cycle			Fig. 3e	Fig. 2d	
Capillary length density			Fig. 5b		

Supplementary Text 5.1. Novelty statement

A variety of OCT imaging and image-processing techniques have been developed, adopted, and integrated for the presented framework. It ranges from reproduction of published methods (e.g., blood flow measurement from Doppler OCT data) and novel application of existing concepts (e.g., betweenness of a capillary network as adopted from graph theory) to development and validation of novel methods (e.g., the deep-learning method for capillary RBC flux measurement; Figs. 5.4a and 5.4b). But more importantly, the major novelty of the presented work lies in the integration of these existing and newly developed techniques into a single framework which has enabled to produce new types of information that otherwise would be highly challenging to obtain if not impossible (e.g., Figs. 5.5c and 5.5d).

Supplementary Text 5.2. Performance of the deep learning-based capillary RBC flux measurement.

Our CNN was also designed to provide a measure of uncertainty in each prediction, allowing us to filter out high-uncertainty predictions in further analysis. Even without this uncertainty filtering, the CNN outperformed the traditional peak-counting method when tested on data unseen by the CNN during training (Fig. 5.4a; the slope is closer to 1, and R^2 is higher). When filtering out predictions with the lowest 20% confidence, the error became both smaller (narrower distribution) and less biased (the mean closer to 0 RBC/s) even for higher flux values (Fig. 5.4b).

Supplementary Text 5.3. Other challenges and opportunities related to the presented methods.

Differences in image quality and brightness may be unavoidable between imaging sessions across either different animals or ages. But many of the results obtainable by the presented methods, if not all, will offer true reflections of underlying physiology. Those image quality differences can originate from many technical and biological factors, including the angle of the beam with respect to the cranial window, the position of the focal plane, as well as the formation of scar tissue. But the presented results support robustness against a potential issue of degrading image quality. A degradation of imaging quality would lead to a loss of resolution and thus result in an apparent increase in the measured diameters of pial and penetrating vessels, but the observed results were opposite (Figs. 5.1e and 5.2d-5.2e).

Another related challenge may be large variations in measurements between animals and/or ages. If one compares absolute values between groups age by age from the presented data, it might suffer from lower statistical power. Such age-by-age comparison does not exploit the analytic advantage of longitudinal data (Supplementary Fig. 5.1). Therefore, careful selection of a statistical method and clear focus of analysis are required. The LME method used here is often preferred for longitudinal data analysis over repeated ANOVA, as LME is relatively robust against missing values and violations of sphericity which increase rates of Type I error [8, 9].

Another potential challenge in robustness is that the absolute values of diameters obtained from OCT angiogram (OCTA) may be inaccurate because the mechanism of OCTA contrast originates from the movement of RBCs through vessels. This may make the OCTA-measured diameter vary with measurement positions along the vessel direction. To minimize such variations, we measured the pial diameter from the mean of several cross-sections extracted along the vessel centerline. But this improves precision, not accuracy. Furthermore, isoflurane used in this study is known to influence vessel diameter. Despite these limitations in accuracy, high precision is often sufficient for many studies that focus on relative differences between

experimental conditions rather than absolute values, like in the presented study. For a study where measurement of the absolute vessel diameter is critical, methods like fluorescent two-photon microscopy may be more suitable although its long-term longitudinal imaging capability has not yet been demonstrated to the best of our knowledge.

In addition to the properties measured in this study, a few related OCT measures have recently been proposed by others, and they can be readily added to the presented framework of methods. For example, capillary stalling has been found to play a role in pathology development in AD by a two-photon microscopy study [10] and then demonstrated to be measurable by OCT [11]. Also, it may be possible and interesting to study whether and how shear-induced diffusion of RBCs as measured by OCT [12] alters with aging.

A direct methodological parallelism is another interesting potential of the presented methods. The OCT upon which the presented methods are based is already being widely used in clinical ophthalmology. Under the assumption that microvascular degenerations appear both in the brain and retina, some preclinical findings enabled by the presented methods may be directly applicable to human studies. In particular, a recent study suggests that cerebral microvascular degeneration may be one of the etiologies, independent of A β , in human ApoE4 AD [13]. ApoE4 accounts for a larger AD population than the familial AD for which the 3xTg model used in the present study was designed. Therefore, if the presented methods are applied to the ApoE4 AD model and produce a similar type of findings demonstrated here (e.g., the betweenness alters the earliest among capillary structural properties), and if such properties are measurable from human retinal OCT images, it would provide unprecedented potential for non-invasive, inexpensive, and imaging-based biomarkers for preemptive prediction of AD. Furthermore, such an application of the same method to both human and mouse will allow us to directly test any

preclinical findings, a kind of ground truth comparison to animal models typically unavailable with most methods.

Supplementary Text 4. Argument about the used 3xTg AD model.

First, it should be noted that this study aims to demonstrate the described technical capability, not directly answer biological questions. We chose the specific model because it quickly develops AD pathologies, since no studies have shown how long on the order of months the OCT methods can repeatedly and robustly image the cortex in the same animal through a chronic cranial window. Nevertheless, the results presented here may provide some insight to the roles of cerebral microvascular degeneration (CMD) in the development of AD, or at least showcases what type of findings will be possible when applied to appropriate models of broader age-related neurodegenerative diseases.

Since no rodent model fully captures the pathophysiology of human AD, a clear understanding of the strengths and weaknesses of each of the various AD models is required for proper interpretation [14]. Compared with some models like ApoE4 knock-in mice [15], the selected 3xTg model is not designed to directly develop CMD but focuses more on A β and tau pathologies [16, 17]. However, CMD interacts with A β accumulation; A β often deposits on the vessel wall, leading to vascular dysfunction, while hypoperfusion accelerates A β accumulation [18]. Transgenic mice overexpressing the A β precursor protein have a profound and selective impairment in endothelium-dependent regulation of the neocortical microcirculation [19]. Therefore, vascular alterations observed from the 3xTg model may indicate either A β -associated vascular pathophysiology or independent CMD as a potential etiology of AD continuum [20, 21].

Supplementary Text 5. Differences in the age of significance between arteriolar/venular diameter/flow.

The differences in the age of significance observed in Fig. 5.5c do not necessarily mean that arteriolar flow, for example, did not change while its diameter altered in early ages. As defined in the main text, the age of significance rather indicates the age at which a vascular alteration becomes pathological (different from WT, while the alteration can occur in both AD and WT), not indicating when the arteriolar flow becomes different from its baseline value (11 WOA). In fact, both arteriolar flow and diameter decreased in early ages (Figs. 5.2d and 5.2g). The difference in the age of significance only indicates that the alteration in the arteriolar diameter became pathological before the arteriolar flow alteration became pathological.

Supplementary Text 6. Implication of the shortest cycle and betweenness result.

The result of these two independent topological measures (shortest cycle and betweenness, Figs. 5.3e and 5.3g) likely spot an identical type of CMD or at least different CMDs that later result in identical vascular vulnerability. The shortest cycle quantifies redundancy in a capillary network, while the betweenness measures the importance of each vessel to the connectivity of the whole network. A larger shortest cycle value means that a network is less redundant, while a higher mean betweenness implies that the network relies more on its subset of nodes (Supplementary Fig. 5.4). Disruption of flow in high-betweenness capillary vessels could have wide reaching effects (unless a physiological shunt of blood flow is established). In our results, both the shortest cycle and the mean betweenness increased with aging in AD, likely both indicating a higher degree of susceptibility to vascular insult than WT.

Supplementary Text 7. Interpretation of capillary flow result.

The pattern of blood flow through a capillary vessel network plays an important role in the energy supply regulation of the brain and its malfunction in diseases [22-25]. In our results, RBC flux gradually increased with aging in WT mice, being consistent with previous findings from WT rats [26] although the previous study measured RBC flux at only two ages in a non-longitudinal manner. AD mice also showed an increase in RBC flux but at a higher rate (6.0% versus 2.1% per month). We also observed reduction in the COV of RBC flux in AD mice (i.e., more homogenized capillary flow pattern). Whereas higher RBC flux and lower flow heterogeneity in capillaries are generally associated with higher oxygen extraction [22, 27], the observed increases in RBC flux and its homogeneity with aging might be due to a compensatory mechanism for capillary vessels to suppress negative effects of the observed, earlier reduction in arteriolar and venular flow, similar to an adaptation occurring in hypoxia [28]. We speculate that such compensatory mechanisms do not last indefinitely, and thus if we had tracked the capillary flow properties for an extended period, we might have seen a decrease in capillary RBC flux and increase in RBC flux COV at a later age. Further studies are required to test this hypothesis.

5.10.1. References for supplementary information

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6. Conclusions and future directions

This thesis demonstrates how OCT can be leveraged to obtain quantitative information related to cortical tissue and vasculature. This is imperative for hypothesis testing in an automated and objective manner, as well as for the analysis of large datasets. Such quantitation is crucial to the rigor of scientific inquiry, but also makes it possible to leverage this technology for new applications. For example, the attenuation coefficient of cortical tissue makes it possible to define diagnostic criteria in identifying cancerous vs. noncancerous tissue, thereby guiding cancer resection [1–3]. Towards this goal, we demonstrate how the attenuation coefficient can be determined more accurately than the standard method by estimating the 2D position of the focal plane, which may be curved or tilted within the sample. This is especially important for high-magnification objective lenses, which have small depth of fields, thereby extending the applicability of this metric. We demonstrate our improved method on human glioblastoma xenograft in a murine model, which shows higher attenuation coefficients in cancerous regions.

In addition to structural information, OCT can be used to obtain 3D visualizations of perfused vessels [4,5]. Study of the vasculature is important for the understanding of Alzheimer's disease, stroke, neurovascular coupling, among others. OCT angiography is gaining traction for neuroscience research due to its label-free capability, penetration depth, and high-speed. However, it has remained challenging to extract quantitative information from 3D angiograms, primarily due to the tails which appear beneath vessel cross-sections due to multiple-scattering [6], and the reduced vessel contrast of vessels which are parallel to the imaging beam [7]. To overcome these limitations, we employed deep learning to enhance, segment and connect vessels in OCT microangiograms. We showed that our proposed solution effectively mitigates projection artifacts, aids visualization of vertical capillaries, and fills erroneous gaps between connected capillaries. We made these tools freely available to any researcher as a toolbox,

allowing users to extract the underlying vascular graph from their datasets and determine angioarchitectural properties such as vessel diameter, branching order, tortuosity, and length.

Next, we turned our attention to quantifying capillary flow, which is traditionally accomplished by counting the number of peaks in the OCT intensity per unit time [8,9]. However, this method is difficult to implement with confidence due to the underestimation of RBC flux at high flux values, the need for a user parametrization, and the inherent uncertainty in identifying “true” RBC passages even when assessed visually [10]. To overcome these challenges, we made use of data which was simultaneously acquired using OCT and two-photon microscopy, the gold standard for RBC flux measurements. This allowed us to train a deep neural network to predict RBC flux, as well as provide a measure of uncertainty in its predictions. We also exploited this dataset to gain insights into the structure of the distributions of peak heights, peak widths, and inter-peak times which underlie the time-series data. This allowed us to develop a validated framework for simulating RBC passages, and further train our CNN to be more robust.

We then combined these methods to longitudinally track 24 vascular metrics in the same animals to study the differential onset of vascular changes that model Alzheimer’s disease mechanisms. This integrated set of in vivo imaging and image-processing made it possible to study the morphology, topology, and function of the cortical vasculature across all scales from the large surface pial vessels to the capillaries which make up the microvascular bed. Such information-rich, multi-feature analyses make it possible to construct a timeline of vascular degeneration in Alzheimer’s disease and identify therapeutic targets. Furthermore, the same methods can be extended to other vascular phenomena such as stroke, traumatic brain injury, and vascular dementia and provides a means to monitor long-term vascular responses to therapeutic intervention.

The possibilities and future directions of this imaging modality are vast. Future studies could look toward further integrating OCT capabilities, such as neuronal visualization using optical coherence microscopy [11,12], and cellular viability using dynamic light scattering OCT [13,14]. The methods proposed in this thesis could also possibly be applied to visible-light OCT, which enables the optical determination of blood oxygenation achievable thanks to the contrasting optical absorption spectra of oxy- and deoxy-hemoglobin [15,16]. This would enable observational studies between vascular network structure and oxygenation, as well as the validation of models aiming to relate RBC flux to tissue oxygenation [17]. Combining microangiogram, Doppler OCT, and RBC flux data would also enable the validation of blood flow models, which can then be manipulated to understand the exact contribution of a single vascular alteration to the overall system. This is difficult (if not impossible) to accomplish experimentally, opening a new avenue to deepen our understanding of the vasculature in health and disease.

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