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UNIVERSITY OF CALIFORNIA SAN DIEGO

Imaging, Construction, and Analysis of Whole Mouse Brain Vascular Connectome

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Physics

by

Xiang Ji

Committee in charge:

Professor David Kleinfeld, Chair
Professor Patrick Diamond
Professor Yoav Freund
Professor Alex Groisman
Professor Marcos Intaglietta
Professor David Traver

2023

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University of California San Diego

2023

DEDICATION

To the universe.

EPIGRAPH

Physics was a point of view that the world around us is, with effort, ingenuity, and adequate resources, understandable in a predictive and reasonably quantitative fashion.

John J. Hopfield

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ABSTRACT OF THE DISSERTATION

Imaging, Construction, and Analysis of Whole Mouse Brain Vascular Connectome

by

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Doctor of Philosophy in Physics

University of California San Diego, 2023

Professor David Kleinfeld, Chair

The vascular system maintains brain homeostasis optimized for the dynamic computation of neurons. The architecture of the vascular network is fundamental to its functionality as a vital transport system. Nonetheless, comprehensive studies into the structure of the entire brain vascular network and its role in homeostatic regulation have been limited, largely due to the multiscale complexity of the system. This thesis presents novel experimental and computational methodologies developed to construct and analyze mouse brain vascular connectome.

We developed techniques to completely label and image whole mouse brain vasculature at sub-micrometer resolution. An efficient computational pipeline was developed to convert immense raw data into a microvascular connectome, the spatial graph representation of the

network documenting the position and radius of 6 million interconnected vessel segments in a trillion-voxel space, with 99.9% connectivity accuracy.

Utilizing this dataset, we analyzed the structure of the vascular network across brain regions. Topological analyses reveal a common network connection pattern across the brain, leading to a universal structural robustness rooted in percolation transition. Systematic quantification of network orientation preference reveals brain regions with striking microvascular anisotropy, which bears implications for interpreting functional magnetic resonant imaging (fMRI) data. By combining biophysical analysis with numerical simulations, we deduced a formula connecting resting-state metabolism rate to network density and further predicted a common value of maximum tissue oxygen tension across the brain. Extending beyond static structure, perturbation analyses quantified the impacts of single vessel dilation, constriction, and obstruction on local blood flow and tissue oxygenation.

Toward constructing vascular connectomes suitable for large-scale flow simulations, we further explored the use of nonlinear optical techniques to image the entire brain vasculature within the cranium at sub-micrometer resolution. High-resolution two-photon and second-harmonic imaging were combined with online processing to define ablation trajectories and parameters for different tissues. Spatiotemporally focused femtosecond pulses were applied for precise and efficient material removal. This entire process was automated through custom-built control software to ensure reliable multi-day operation. This system enabled a detailed examination of the complex vascular connection between the brain and the skull, vital for modeling cerebral blood flow.

Chapter 1

Introduction

The intricate blood vessels that wind through the living tissue of organisms are as ancient as the hills and as vital as the breath of life itself. In the case of mammals, and particularly within the context of the mouse brain, this vascular network is a marvel of biological engineering, a testament to the evolutionary processes that have shaped life on Earth. This doctoral thesis delves into the realm of the vascular labyrinth, aiming to digitize and illuminate the complexity of the entire mouse brain vascular network through advanced imaging, meticulous reconstruction, and comprehensive analysis.

1.1 The circulatory system

The circulatory system, a hallmark of the animal kingdom, represents one of the most significant evolutionary adaptations to life on Earth. From the simplest invertebrates to the most complex mammals, the evolution of this system has been pivotal in enabling the incredible diversity of life.

The evolution of the circulatory system was primarily driven by the laws of chemistry and physics. In organisms ranging from unicellular to simple multicellular life forms, the essential exchange of oxygen, nutrients, and carbon dioxide with their surroundings occurs through the process of diffusion. While diffusion is an energetically favorable mechanism, it is limited by its slow pace and minimal range of efficacy. The distance over which particles diffuse in three-

dimensional space over time t can be estimated as $\sqrt{6Dt}$, where D is the diffusion coefficient. Specifically, the diffusion coefficient of oxygen in water is approximately $2.4 \times 10^{-9} \text{ m}^2/\text{s}$, which equates to a mere 0.12 mm in one second - a timeframe pertinent to metabolic activities. For an organism on the scale of $\mathcal{O}(L)$, the rate of exchange J for a diffusing substance is proportional to the surface area $\mathcal{O}(L^2)$, while the metabolic demands scales as body volume $\mathcal{O}(L^3)$. An increase in body size has been a major evolutionary trend among animals. Microorganisms optimize their surface-to-volume ratio by developing convoluted surfaces or adapting elongated shapes, and strategically placing metabolically active cells close to the organism's periphery. Despite these adaptations, their effectiveness is fundamentally limited by physics and geometry. Once an organism grows beyond a certain size, its surface area is insufficient to support its metabolic requirements through diffusion alone. This limitation necessitates the evolution of more complex internal transport and exchange mechanisms (circulatory system) to cells throughout the organism's body.

Current research indicates that the blood vascular system emerged first in the common ancestor of triploblastic organisms over 600 million years ago[81]. All vertebrates have evolved from this ancestor and thus constructed on a shared structural blueprint[65]. All vertebrates' cardiovascular system is distinguished by a network of enclosed vessels, each lined with endothelial cells and typically surrounded by smooth muscle cells or pericytes. This endothelial lining, whose origins can be traced to approximately 525 million years ago, is characterized by a layer of epithelial cells exhibiting basoapical polarity, intercellular junctions, and connecting to the basement membrane. Despite the presence of contractile vessels, the propulsion of blood in vertebrates is driven by a centralized muscular organ - the heart. Furthermore, most vertebrates are equipped with a lymphatic system, a critical auxiliary network that collects and returns interstitial fluid to the bloodstream, maintaining fluid balance and playing an essential role in immune response.

As the vascular system has evolved, its role has expanded and diversified to fulfill the specialized requirements of different organs within the body. This adaptation is a testament

to the vascular system's dynamic nature, as it not only performs fundamental tasks such as transporting nutrients, oxygen, and carbon dioxide but also supports more specialized functions tailored to the needs of various tissues. For instance, the skin's vascular network is finely tuned for thermoregulation, efficiently dissipating heat or conserving warmth as needed. In the kidneys, the system has specialized to facilitate ultrafiltration, a critical process for maintaining fluid and electrolyte balance. The vascular framework also supports defense mechanisms, optimizing the delivery of clotting factors to prevent hemorrhage and circulating immune cells to fend off infections. Furthermore, it plays a crucial role in whole-body integration, efficiently disseminating hormones to coordinate a myriad of physiological processes. Such functional diversification illustrates the vascular system's dynamic evolution, evidencing its central role in the physiological specialization across mammalian organ systems[111, 100].

1.2 Mammalian brain vascular system

The remarkable adaptability of the mammalian vascular system is perhaps most strikingly illustrated in the brain, where it has undergone sophisticated specialization to support the complex brain physiology. Despite accounting for only approximately 2% of the body's mass, the brain receives about 15% of the cardiac output. The intense metabolism of glucose [3, 52, 23] is sharply contrasted with the lack of local metabolic substrate reserve, necessitating a large and stable supply of blood flow[46]. Brain vasculature responds to the spatially and temporally varying metabolic demands through structural adaptation and modulation on multiple scales. A matured brain vascular network exhibits structural heterogeneity across brain regions[8, 19, 123] with different cellular compositions[121] and metabolism[130], while adapts to chronic oxidative metabolic changes by angiogenesis[8, 79]. In response to the spatiotemporal variation of neuronal activities, a multiplicity of neuronal and vascular mechanisms further modulate the structure of the vasculature to coordinate blood flow supply to different regions[2, 21, 39, 61]. The structure of the vascular network plays a central role in determining the physiological functions of this

critical system.

The brain's vascular network is not only pivotal to meeting its energy demands but also to maintaining a finely tuned environment for neuronal functions through the blood-brain barrier (BBB). Present in all organisms with a well-developed central nervous system (CNS), the BBB consists of endothelial cells that form the innermost wall of vessels and capillaries, enveloping astrocyte end-feet, and supportive pericytes. Unlike their counterparts in other organs, these endothelial cells lack fenestrations, have more extensive tight junctions, and engage in less pinocytotic vesicular transport. Supporting cellular components including astrocytes and pericytes are integral to the BBB's induction, maintenance, and function. This barrier defends against the fluctuation of ionic concentration and potentially neuroexcitatory amino acid in the bloodstream, which can vary due to various physiological or pathological conditions. Moreover, the BBB blocks neurotoxic substances in the blood plasma, both endogenous and exogenous, thus safeguarding the CNS, which typically exhibits limited regenerative capabilities. This protection is crucial in preventing the premature declination of CNS function, a system vital to an organism's overall well-being and functionality[1, 5].

The overall hierarchical structure of the mammalian brain's vascular network begins with major arteries that enter the brain through bony holes in the skull base, an entry that is protected and structured to facilitate the smooth passage of these vital vessels. The main suppliers are typically the paired internal carotid arteries and the basilar artery formed by the vertebral arteries. These arteries are part of the Circle of Willis, an arterial ring that provides redundancy and equalizes blood flow to both hemispheres of the brain. Branching from the Circle of Willis, major cerebral arteries, such as the anterior, medial, and posterior cerebral arteries, extend across the brain's surface, each supplying broad territories of the brain. From the pial arterial network, penetrating arteries delve into the brain parenchyma, giving rise to a dense network of capillaries. The capillary beds are the major place for the exchange of gases, nutrients, and metabolic waste. Blood flows to these beds are precisely regulated in response to neuronal activity. This phenomenon is known as neurovascular coupling, the underlying mechanism of

functional magnetic resonant imaging (fMRI). Once the blood has passed through the capillary network, it is collected by venules and veins. The venous blood is then directed towards the dural sinuses, large venous channels that sit between the layers of the dura mater. Finally, blood exits the brain via the internal jugular veins, completing the cerebral circulation.

1.3 History of studies in vascular system

The historical quest to understand the vascular system, including the intricacies of cerebral circulation, stands as one of the most fascinating chapters in the annals of biology and medicine. This journey of discovery has been punctuated by pivotal findings that have incrementally illuminated the complex workings of the circulatory network, stretching from ancient times to the sophisticated research of the modern era.

Our understanding of the systemic circulatory system began with ancient civilizations, which proposed various theories about the role and function of blood and vessels. However, it was not until the 2nd century AD that Galen of Pergamon would articulate a comprehensive, albeit flawed, model of the circulatory system, postulating that blood is produced in the liver and consumed as fuel by the body.

The paradigm-shifting moment occurred in 1628 when William Harvey published "De Motu Cordis", detailing his revolutionary discovery of the circulatory nature of blood flow, describing that the heart operates as a pump to circulate blood throughout the body. Harvey's work laid the foundation for subsequent research into vascular and cardiac physiology.

Investigation into brain vasculature began in earnest in the centuries following Harvey's breakthroughs. Detailed studies in the 18th and 19th centuries, by anatomists like Thomas Willis, after whom the Circle of Willis is named, began to unravel the complexities of the cerebral vascular system. In the 19th and 20th centuries, the development of microscopes allowed for the exploration of smaller vessels[89, 19] and the discovery of blood brain barrier.

The study of microcirculation took a great leap forward in the work of August Krogh

in the early 20th century. Krogh's investigations into capillary physiology[62], for which he was awarded the Nobel Prize in Physiology or Medicine in 1920, elucidated the mechanisms by which capillaries are recruited to regulate blood flow in skeletal muscles. These studies laid the foundation for understanding the dynamic regulation of blood flow in response to tissue demands - a principle also crucial to brain function.

With the advent in modern technology such as computed tomography (CT), magnetic resonance imaging (MRI), ultrasound, and advanced optical and electron microscopy techniques, the latter part of the 20th century till now have seen an explosion of detailed information about the cerebral vasculature and blood flow across spatiotemporal scales. These advances have been accompanied by a deeper understanding of the molecular and cellular mechanisms governing the development, maintenance, and pathology of the brain vasculature. Research has increasingly focused on the interactions between the vascular system and the neurological function, exploring the role of blood vessels in neurodegenerative disease, stroke, and other conditions[115, 95, 46, 47].

The history of studying the vascular system reflects a trajectory of increasing complexity and precision, from the systemic insights of Harvey to the microvascular focus of Krogh. This thesis stands on the shoulders of these scientific giants, aiming to connect the systematic physiological functions on the whole brain scale with the intricate microscopic structures of the vascular network. As we embark on this journey, we acknowledge the contribution of many minds and hands that have toiled in this field before us. It is with a sense of deep respect and burning curiosity that we proceed to unfold the following chapters.

1.4 Vascular connectome

The maxim "structure determines function" has long been a cornerstone principle in biology that is particularly pertinent to the vascular system. This system's evolution was primarily driven by the need to overcome the biophysical limitations of diffusion, with blood flow propelled

by biomechanical forces. Alternations to the network structure, whether through angiogenesis or the pruning of vessels, have direct implications for diffusion sources and the trajectories of blood circulation. Moreover, the coordinated regulation of vessel diameters fine-tunes fluid resistance and, in turn, the distribution of flow. In light of this, a "vascular connectome" — essentially a comprehensive "plumbing diagram" that details the position, radius, and connectivity of every vessel segment — would be invaluable for quantitative studies of the vascular network.

Despite the potential insights such connectome promises, its realization has been fraught with challenges, largely due to technological limitations in data acquisition and subsequent analysis. As a matter of sample preparation, all vessels must be fully labeled down to the finest capillaries. Additionally, morphological alternations induced by fixation and embedding processes must be minimized and quantified. The vascular network's hierarchical organization extends across spatial scales, from the macroscopic breadth of organs down to the microscale diameters of capillaries. Capturing the entirety of this complex structure within an organ, or across an entire organism, demands imaging at micrometer resolution in three dimensions. Given the steep dependence of flow resistance to vessel radius[109], advancing lateral resolution to sub-micrometer level becomes essential for precisely determining capillary dimension. Such high-resolution imaging inevitably generates an immense volume of raw data, which necessitates a robust, specialized computational pipeline for its processing and interpretation.

The endeavor to construct detailed vascular connectome can be traced back to 1971, with pioneering work by Drs. Intaglietta and Zweifach at UC San Diego. Their study on rabbit omentum resulted in the first vascular connectome with clearly defined arterial inputs, venous outputs, and 50 to 100 capillary segments[49]. Subsequent studies extend this work to the cat[71] and rat[96, 98] mesentery, creating connectomes encompassing hundreds of vessels. These early studies primarily utilized bright-field microscopy to image quasi-two-dimensional vascular networks *in vivo* for studying network structures and blood flow.

The turn of the millennium marked a significant upscaling in these endeavors, driven by advances in both imaging and computational analysis. Cassot et al used confocal laser microscopy

to image ink-injected human brain cortical vasculature at $1.22 \times 1.22 \times 3 \mu m^3$ resolution and reconstructed networks with about 15,000 segments. Risser et al [104, 103, 102] harnessed synchrotron X-ray micro-tomography (μCT) to visualize primate cortical vessels at $1.4 \mu m$, reconstructing networks comprising around 50,000 segments. Rodent cortical vasculatures have since been mapped at similar scales, utilizing all-optical histology[120] at $1 \mu m$ resolution[6], and synchrotron μCT at $0.7 \mu m$ resolution[101]. These local vascular connectomes have been applied for quantitative analysis of brain blood flow patterns[108, 40, 75], hemodynamics[40, 107], tissue oxygenation[124, 33] under normal or pathological conditions[6, 20, 27], and for interpreting high-resolution functional magnetic imaging signal[4, 35].

Recent advancements in brain-wide vascular imaging have marked significant progress in the field[59, 117, 133]. These recent research, in conjunction with previous studies[19, 8], have revealed the diverse vessel density across brain regions and underscored the profound impact of major events, such as strokes, on vascular remodeling[59]. While highly informative, current reconstruction techniques have yet to fully capture the intricate connectivity among vessels throughout the brain, nor do they accurately measure capillary diameters. These limitations hinder comprehensive topological and geometrical analysis of network structure and preclude the possibility of conducting large-scale flow simulations. To overcome these challenges, we developed novel experimental and computational methodologies to construct whole mouse brain vascular connectomes with unprecedented completeness and precision.

Chapter 2

Imaging Whole Mouse Brain Microvascular Network

2.1 Labeling brain vascular network

Complete filling of the vasculature is a balance between sufficient pressure to fill all vessels but not rupture vessels. We used a variant of a past technique [121] to fill vessels with gelatin and fluorescein-conjugated bovine serum albumin and then cross-link these constituents to lumen proteins. As a control, we compared the same volume of labeled vessels in the cortex after perfusion in the living animal. We found seven partially unfilled short segments among 2835 vessel segments in 15 volumes across 3 animals, i.e., 99.8% of all microvessels had no discernable gap and the 0.2% of vessels had a gap of less than 10 μm (Figure 2.1).

2.1.1 Vascular fluorescent-gel fill-based labeling

Adult male mice between 50-80 days of age were prepared for whole-brain vascular labeling. Mice were injected IP with heparin (200U) and then overdosed with isoflurane delivered in oxygen and injected with fatal plus (IP injection). Exsanguination was performed by transcardial perfusion of 60-150 mls Oxygenated Ringers Carbicarb (37 °C) with 10U/ml of heparin added to the perfusate. Ringer's perfusate was delivered from a peristaltic pump, set at a pump rate of 30 ml/minute, through a blunted 18 g cannula that was inserted through an incision made in the left ventricle; the right atria was snipped to provide an exit for exsanguinated blood.

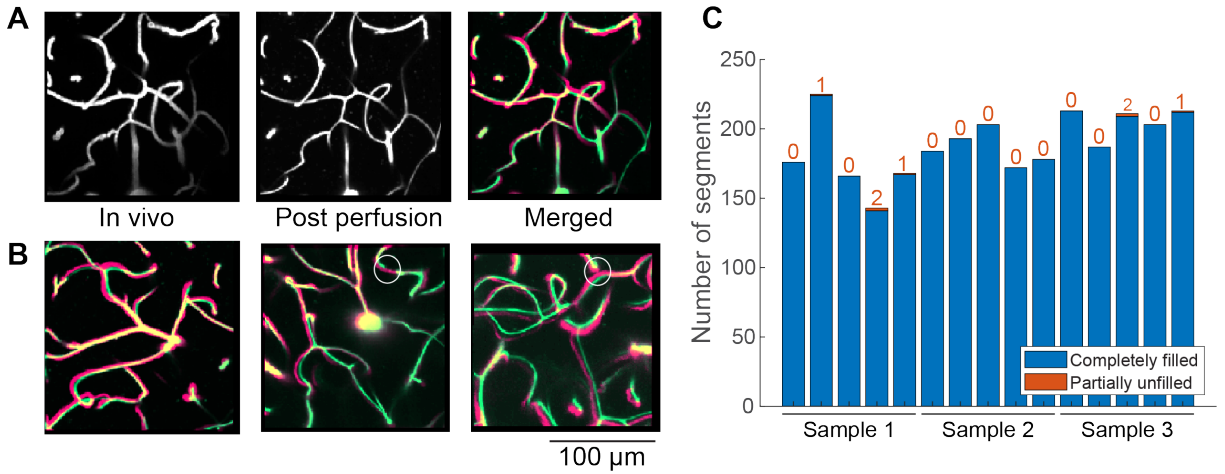


Figure 2.1. Labeling quality quantification (A) 60 μm thick maximum intensity projection of in vivo vascular image, registered post perfusion image and their overlay (Red channel: in vivo; Green channel: post perfusion; Blue channel: average of in vivo and post perfusion image grayscale value; the same as panel B). For in vivo images, stronger absorption in the pial vessels may shadow the microvessels underneath and lead to uneven signal-to-noise levels across the image. (B) 60 μm thick maximum intensity projection of merged in vivo and post-perfusion vascular images. The first image shows complete labeling. The second and third images show two rare examples of partially unfilled vessel segments (circled). (C) Cerebral vascular labeling completeness quantification in 15 volumes from 3 brain samples. Each volume is about $180 \times 180 \times 400 \mu\text{m}^3$. 7 out of 2835 vessel segments were found to be partially unfilled.

Exsanguination was judged complete by blood clearing of sternal vessels and of the dental pulp. Following the exsanguination step, the head of the mouse was tilted down and the descending aorta was clamped. Fluorescently labeled gelatin (10% porcine gelatin- 0.4% FITC-BSA in PBS-azide) was then administered from a glass syringe-tubing assembly connected to a blunted perfusion cannula that was inserted into the original incision in the left ventricle. Towards the end of the perfusion, as the perfusion cannula was withdrawn from the left ventricle, a small chip of dry ice was used to rapidly freeze the heart to minimize leakage of the warm gelatin through the perfusion incisions. The entire body was then covered with wet ice for 1 hour to cool the intravascular gelatin. The brain was immersion fixed in the skull by placement in cold paraformaldehyde/PBS for 48 h. After a cold PBS wash step (48 h), the brain was removed from the skull under a fluorescent dissection microscope. The extracted brain was stored in cold PBS.

Gelatin solution was prepared by first blooming the porcine gelatin powder with cold PBS and then microwave-heating it to temperatures that were not in excess of 60 °C. FITC-BSA in PBS-azide was added to liquefied gelatin that had been cooled to 37 °C. While the resultant solution is typically clear after coarse filtration, microscopic examination of the fluorescent gelatin revealed bubbles and occasional particulates. To minimize these defects, the liquefied gelatin was sterile-filtered after warming in a 47 °C water bath. Residual air bubbles were then removed by suction of the surface after centrifugation (3000 g – 2 min). The filtered labeled gelatin was held in a 47 °C water bath and transferred to the warmed glass syringe-tubing assembly just before use.

2.1.2 Labeling completeness quantification

Chronic cranial window preparation

Adult (more than 12 weeks old) male wild-type mice (C57BL/6J) were anesthetized with isoflurane using a vaporizer (Ohmeda, Isotec 4), 3% (v/v) in oxygen for induction and 1 - 2% (v/v) for maintenance. Body temperature was maintained at 37°C by a heating pad with feedback control during anesthesia. The animal was given analgesic buprenorphine (s.c., 0.1

μg per g body weight) and placed in a stereotaxic frame. The scalp and the periosteum on the parietal and occipital plates were removed. A 3-mm diameter craniotomy was made over the right somatosensory cortex with the centroid at 1.5 mm posterior to Bregma and 2.5 mm lateral from midline. Dura was left intact. A single piece of 3-mm round coverslip (no. 1, 170- μm thick) was then embedded in the craniotomy and sealed around the edge with cyanoacrylate glue (Loctite, no. 401). Meta-bond (Parkell) was further applied around the edge of the coverslip to reinforce stability. A titanium headbar was glued onto the skull with Meta-bond (Parkell) for head fixation during *in vivo* imaging. The remaining exposed bones were covered with dental acrylic (Lang Dental) to ensure stability. All experimental procedures on our animals were in accordance with the Guide for the Care and Use of Laboratory Animals and have been approved by Institutional Animal Care and Use Committee at University of California, San Diego.

***In vivo* vascular imaging**

Imaging was performed 7 days after the cranial window surgery. Habituation training for head fixation was performed for one hour daily for 3 days prior to imaging. A 5 % (w/v) solution in physiological saline of fluorescein isothiocyanate-dextran (2 MDa; Sigma no. 52471) was prepared for vascular labeling. Before imaging, mice were briefly anesthetized with isoflurane to label the lumen of blood vessels via a retro-orbital intravenous injection of 50- μL fluorescein isothiocyanate-dextran solution. The cranial window was carefully aligned to be perpendicular to the objective axis using a goniometer stage (Thorlabs, GNL20). Mice stayed awake during imaging.

In vivo vascular imaging was performed under a previously developed two-photon microscope [72] with Galvo x replaced by a resonant scanner (Cambridge Technology, CRS 8 kHz). The Galvo y (Cambridge Technology, 5-mm mirror 6215H) remained the same. A tunable femtosecond laser (Coherent, Chameleon Discovery) was set to 920 nm, and the imaging power at post-objective was less than 100 mW in all *in vivo* experiments within 500 μm depth. Vasculatures were imaged at $0.2 \times 0.2 \times 0.94 \mu\text{m}^3$ through a 25x objective (Olympus,

XLPLN25XSVMP2, 25X, 1.0 NA and 4-mm working distance), with system-level aberration correction. The microscope was controlled by ScanImage (Vidrio Technologies) running on MATLAB (Mathworks).

After the mice were perfused with fluorescent gel, the brain was left intact and remained inside the skull. A repeat imaging with the same setting was performed through the cranial window at the exact same location on the perfused brain.

2.1.3 Quantification method

The *in vivo* and post-perfusion cortical vascular images used for vessel radius calibration were processed to extract the vessel skeleton. Vessel skeleton was used to compute the similarity transformations from the post-perfusion image to the *in vivo* images. The post-perfusion images were transformed to overlay on the *in vivo* images according to the similarity transform. To facilitate visual inspection, RGB images were synthesized by using the *in vivo* images as the red channel, the post-perfusion images as the blue channel, and the average of the *in vivo* and post-perfusion images as the green channel. Moreover, the reconstructed vessel graph in the post-perfusion images was transformed and added to the green channel (of value 255), while the unconnected endpoints were added to the red channel (of value 255). For each channel, 20 μm moving maximum intensity projection was computed and the max-projection images were further enhanced by saturating the grayscale value of 2.5% of the pixels. Classifier-based graph refinement was not applied to the extracted vessel skeleton, and therefore the position of the unconnected endpoints helped to highlight the potential unlabeled vessels in the post-perfusion image. The unlabeled parts of the vessel segments in the post-perfusion images had signal levels comparable to their immediate background. The image stacks were inspected multiple times and any uncertainties in the filling completeness were verified in the original images. 15 regions of size $180 \times 180 \times 400 \mu\text{m}^3$ from 3 mice (5 regions from each) were inspected.

2.2 Serial two-photon imaging of whole mouse brain

Entire brains (three mice) were then extracted (Figure 2.2A), post-fixed, index matched, and imaged at $0.3 \times 0.3 \times 1 \mu\text{m}^3$ voxel resolution [26]. We roughly estimated the coordinates for the centerlines of small vessels and the lumen of larger vessels as a means to stitch all of the image stacks by a non-rigid transformation (Figure 2.2B). This led to a composite image of the entire brain at $0.25 \times 0.25 \times 1.0 \mu\text{m}^3$ voxel size with optical distortion and local tissue deformation largely eliminated (Figure 2.2C).

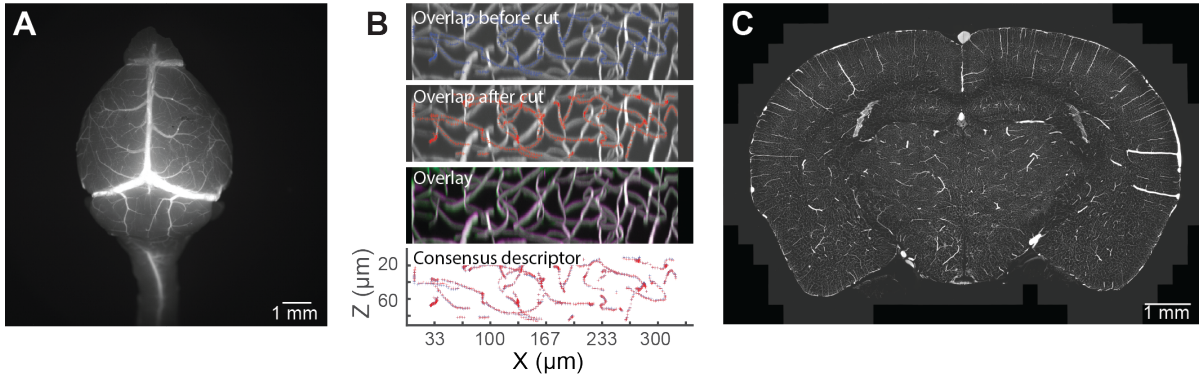


Figure 2.2. Sample preparation, imaging, and image stitching (A) Photomicrograph of a whole mouse brain with vessel lumen filled with fluorescent gel. (B) Vessel skeleton voxels in the overlapping volume of adjacent image tiles in the z-direction were matched to compute the nonrigid transformation for image stitching. The entire brain consists of about 20,000 partially overlapping image stacks. (C) Maximum intensity projection image of a 100-mm-thick slab of the brain after stitching.

2.2.1 Sample Processing and Imaging

All three brains were embedded in 12% (w/v) gelatin and fixed in 4% paraformaldehyde for 12 h. Index matching for optical clarity was achieved by immersing the sample in a solution of 40% DMSO in 10 mM PB with increasing concentrations of D-Sorbitol (up to 40/60 w/v). In *Brain 2* and *Brain 3*, nuclei were stained with 10 μM NuclearID-Red solution (ENZ-52406, Enzo Life Science, Farmingdale, NY). To improve the signal-to-noise level and optical resolution, *Brain 3* was further delipidated after verifying the compatibility of the sample preparation methods. The processed whole brain samples were imaged using a resonant scanner two-photon

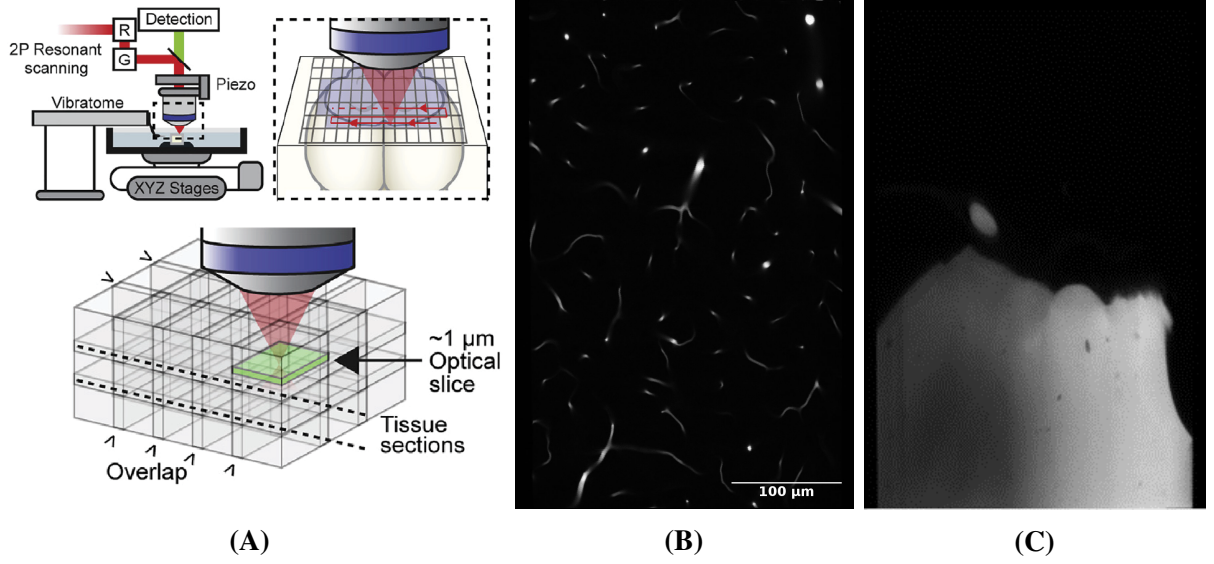


Figure 2.3. Serial two-photon microscopy (A) Illustration of the operation procedure of serial two-photon microscope [131]. (B) A single section of brain microvessel image from a stack. (C) A single section of brain venous sinus image from a stack.

microscope. Imaging was performed using a 40x/1.3 NA oil-immersion objective (#440752, Carl Zeiss, Oberkochen, Germany) at $0.3 \times 0.3 \times 1.0 \mu m^3$ voxel resolution. The surface of the sample was automatically detected and divided into partially overlapping image stacks of size $385 \times 450 \times 250 \mu m^3$. After the $250 \mu m$ thick block face was imaged, the vibratome removed the top $175 \mu m$ of the tissue before proceeding to the next iteration of imaging. Overlap between adjacent image stacks is $25 \mu m$ in the lateral direction and $75 \mu m$ in the axial direction (Figure 2.3). A detailed description of the protocol can be found in [26] [131].

2.2.2 Computational pipeline for image stitching

To generate the integral image of whole brain vasculature with smooth features, about 20,000 3D image stacks need to be computationally stitched together. Direct stitching with stage coordinates might produce numerous artifacts. The main sources of these artifact include (1) tissue mechanical deformation due to vibratome sectioning; (2) tissue expansion due to strain release after mechanical sectioning; and (3) optical aberrations from the imaging system. To address this problem, we adapted the descriptor-based nonrigid 3D image stitching pipeline

[26, 131] for whole brain vasculature images. This involves image segmentation, descriptor extraction, point cloud registration, outlier rejection, deformation field estimation, and image rendering.

Feature extraction

The radius of mouse brain vessels spans two orders of magnitude, from about $2\ \mu m$ for capillaries (Figure 2.3B) to more than $100\ \mu m$ for the venous sinuses (Figure 2.3C). For most of the image stacks containing capillary network uniformly, vessel centerlines (skeleton) are good descriptors for image registration. The vessel centerline can be computed by binarizing the high-resolution image stack with a local adaptive thresholding method, followed by a morphological thinning algorithm. The associated vessel radius was estimated by 2D Euclidean distance transform within the vessel mask. The skeleton voxels are classified based on the number of skeleton voxels in their 26-neighbors. Voxels with 2 neighbors are defined as link voxels and grouped into order lists that correspond to vessel segments. Skeleton voxels in segments longer than $10\ \mu m$ with a median radius no greater than $3\ \mu m$ were selected as descriptors (Figure 2.4). When the cross-section of the major vessels is comparable to the size of the image tile, vessel centerline position cannot be reliably estimated based on local partial information. For these image tiles, we also computed the position of the vessel wall as the descriptor using an approximated 3D canny filter.

Both skeletons and edge voxels in adjacent image tiles were matched by the Coherent Point Drift algorithm and the matched descriptors were used for computing the nonrigid transformation that projects the image tiles into an integrated image in $0.25 \times 0.25 \times 1.0\ \mu m$ voxel resolution[131].

Description matching

Descriptors in the overlapping volumes of the adjacent tiles are matched to estimate the sample deformation and optical distortion. Each tile has 6 adjacent tiles in $-x$, $-y$, $-z$, $+x$, $+y$,

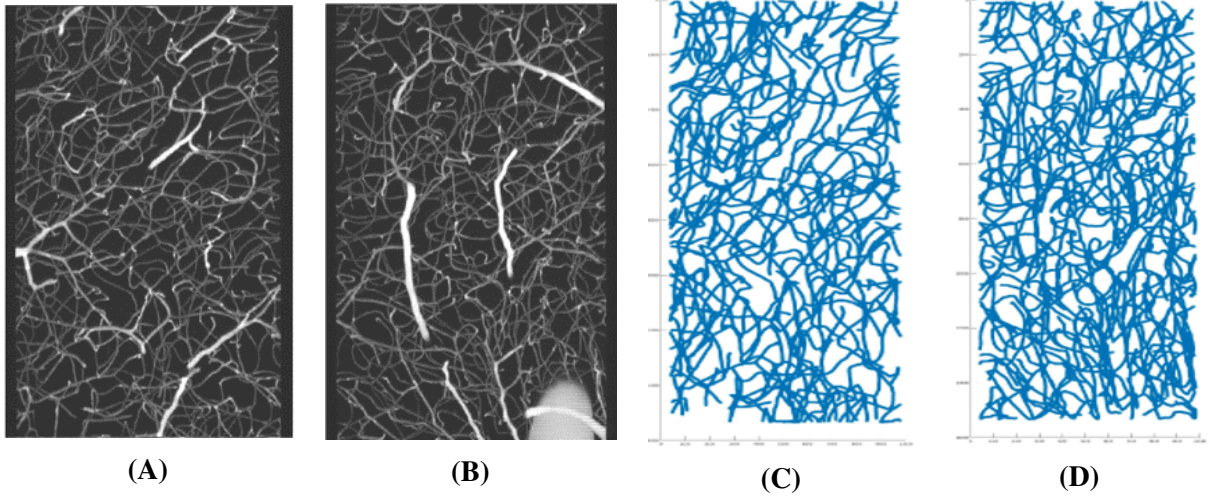
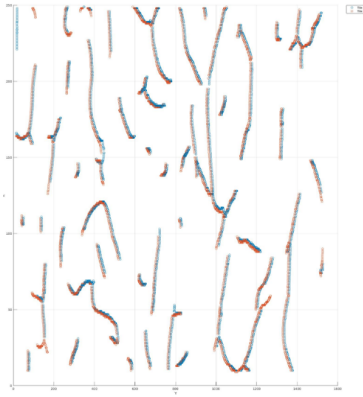
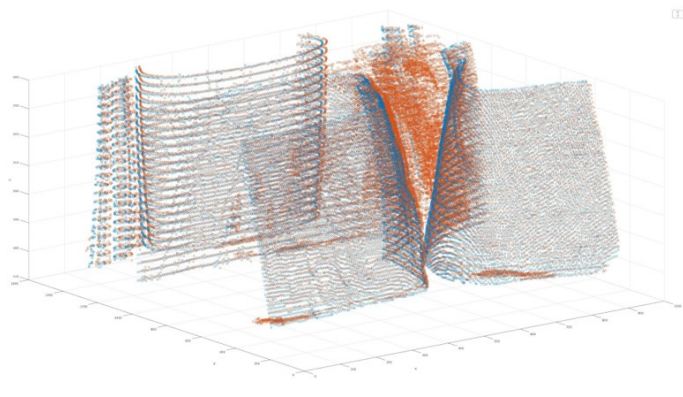


Figure 2.4. Microvessel descriptor extraction (A, B) Maximum intensity projection of a raw image tile along z-direction. Panel B is the x+ adjacent tile of the tile shown in panel A. (C, D) Vessel skeleton as the descriptor extracted from the 3D image tiles in panels A and B, respectively.

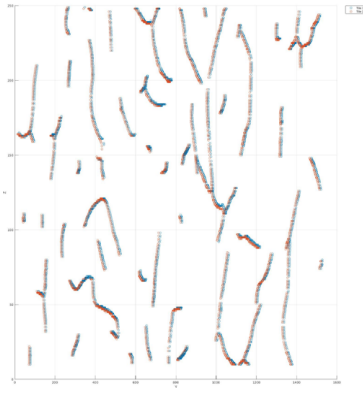
+z directions. The descriptor of one tile is matched to the descriptors of its adjacent tiles in the +x, +y, +z directions. As tiles are acquired in a fixed grid of stage coordinates, these coordinates provide the initial translation displacement vector between the adjacent tiles. Descriptors outside the estimated overlapping volume bounding box are removed (Figure 2.5A and Figure 2.5B). The descriptors from both tiles form two point cloud that can be registered using the Coherent Point Drift (CPD) algorithm[85]. CPD treated one point cloud as the centroids of a mix of Gaussian distributions and found the (non-rigid) transformation that transformed the other point cloud to maximize the posterior probability using Expectation-Maximization Algorithm. After registration, the correspondence between descriptors from two tiles was established by the mutual nearest neighbor after transformation. Falsely matched descriptors were further rejected by assuming the matched skeleton voxels are from a pair of vessel segments (Figure 2.5C and Figure 2.5D). A set of criteria was designed to evaluate the registration result. If the number of qualified matched descriptors is small (e.g. < 1000), the initial translation displacement vector is re-estimated for the next iteration of registration. Displacement estimation was based on the mechanical error from the stage and the vibratome, as well as the overall displacement vector of



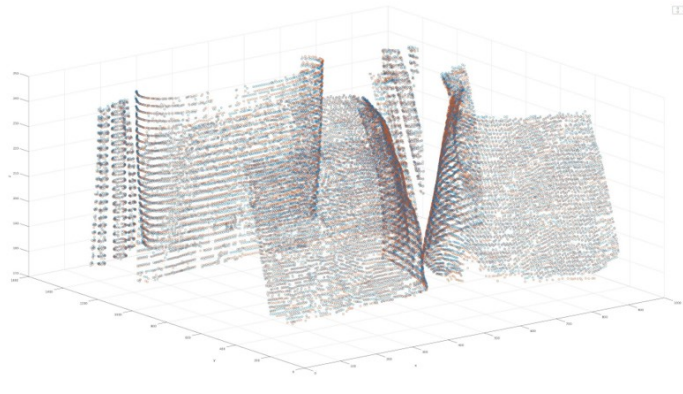
(A)



(B)



(C)



(D)

Figure 2.5. Vessel descriptor matching] (A) Microvessel skeleton descriptors from two adjacent tiles before registration. (B) Vessel wall descriptors from two adjacent tiles before registration. (C) Microvessel skeleton descriptors from two adjacent tiles after registration. (D) Vessel wall descriptors from two adjacent tiles after registration.

the previous iteration of registration. If this procedure still failed to produce satisfactory matching, the image stacks of the pair tiles were used to compute a global translation displacement vector directly using an intensity-based Masked FFT registration [93]. If the resulting vector is not very far from the one estimated based on stage coordinate and the registration has a high correlation coefficient, this displacement vector was then used to initialize another round of point cloud registration. If insufficient matched descriptors are matched after this process, the process terminates.

For most of the tiles containing mainly microvessels, matching skeleton descriptors was usually sufficient to produce statistically robust correspondences. If the tiles were near the surface of the brain where part of the tile contains space in the background, or if the tiles contain large vessels, the matching procedure mentioned above was repeated for vessel wall descriptors. As the CPD algorithm needs to create multiple matrices between pairs of descriptors, the memory cost scale as $\mathcal{O}(NM)$ where N and M are the number of descriptors in the tiles. A typical tile containing mostly microvessels has about 30,000 vessel skeleton descriptors, while tiles that partially contain venous sinuses could have more than 1 million vessel wall descriptors. For distributing the computation, we limit the number of descriptors for CPD to be less than 10,000 for skeletons and 20,000 for vessel walls. Vessel skeleton voxels are grouped into segments and those in the long segment were kept. Vessel wall descriptors were grouped into 3D grids and the center of mass of the descriptors inside each grid cell was used.

Deformation field estimation and rendering

After matching the descriptors in all pairs of tiles, the deformation field was estimated in multiple steps, each allowing for increasing flexibility. To create a seamless image of brain vasculature, especially at the tile edges, we needed to numerically correct both optical distortions from the imaging system and mechanical distortion arising from vibratome sectioning and long-term sample changes. Optical distortions, stemming from potential hardware misalignment and objective distortion, remain consistent regardless of the sample and are expected to be stable over the fortnight of microscope operation. These distortions can be broken down into an overall 3D projection error, alongside a radial distortion characterized by a quadratic function. Once optical distortion is accounted for, we further select descriptors based on residual displacement, assuming the tissue deformation is small and smooth across a span of several millimeters. These selected pairs enabled the computation of the local non-rigid deformation field. We then created and adjusted a grid of control points to counteract the identified deformations.

An efficient computation pipeline was developed to merge the image tiles into a cohesive

space, guided by the adjusted control points ([131]). This pipeline produces a stitched image of a whole mouse brain at $0.25 \times 0.25 \times 1.0 \mu m^3$ resolution, spanning a space with about $30000 \times 40000 \times 15000$ voxels (about 20 TB per channel). For efficient visualization and handling, this image is divided into non-overlapping 3D image tiles and further iteratively downsampled to generate the image pyramid organized as an octree.

2.3 Acknowledgment

Chapter 2 to 4, in part, is a reprint of the material as it appears in X. Ji, T. Ferreira, B. Friedman, Rui Liu, H. Liechty, E. Bas, J. Chandrashekar, D. Kleinfeld, Brain microvasculature has a common topology with local differences in geometry that match metabolic load, *Neuron*, Volume 109, Issue 7, 2021, Pages 1168-1187.e13. The dissertation author was the primary investigator and first author of this paper.

Chapter 3

Computational Pipeline for Constructing Whole Brain Vascular Connectome

We developed an efficient computational pipeline to extract the complete static structural information of the vascular network from tens of terabytes of images. This process starts with dividing the whole brain image into small cubes to facilitate parallel processing. Subsequently, an intensity-based image segmentation algorithm was applied to detect the position of vessels in these cubes. A morphological thinning algorithm is then employed to transform the vessel mask derived from segmentation into vessel centerlines. By amalgamating these centerlines, we constructed the vascular connectome — a spatial graph representation of the vascular network. To further enhance the connectivity accuracy of the connectome, we developed a classifier-based graph refinement approach. This was complemented by a model-based method to estimate the vessel radius with super-optical resolution accuracy. This pipeline produces whole brain vascular connectomes with skeletons positioned at $1\ \mu m$ resolution and vessel radius estimated at $0.25\ \mu m$ resolution.

3.1 Volume decomposition for parallel processing

This pipeline was implemented in MATLAB (2019b) and deployed on a desktop workstation with dual Intel Xeon E5-2687W CPUs (2 sockets, 24 cores), 512 GB RAM, an NVIDIA GeForce GTX 1080Ti GPU, and an NVIDIA GeForce RTX 2080 GPU. To efficiently handle

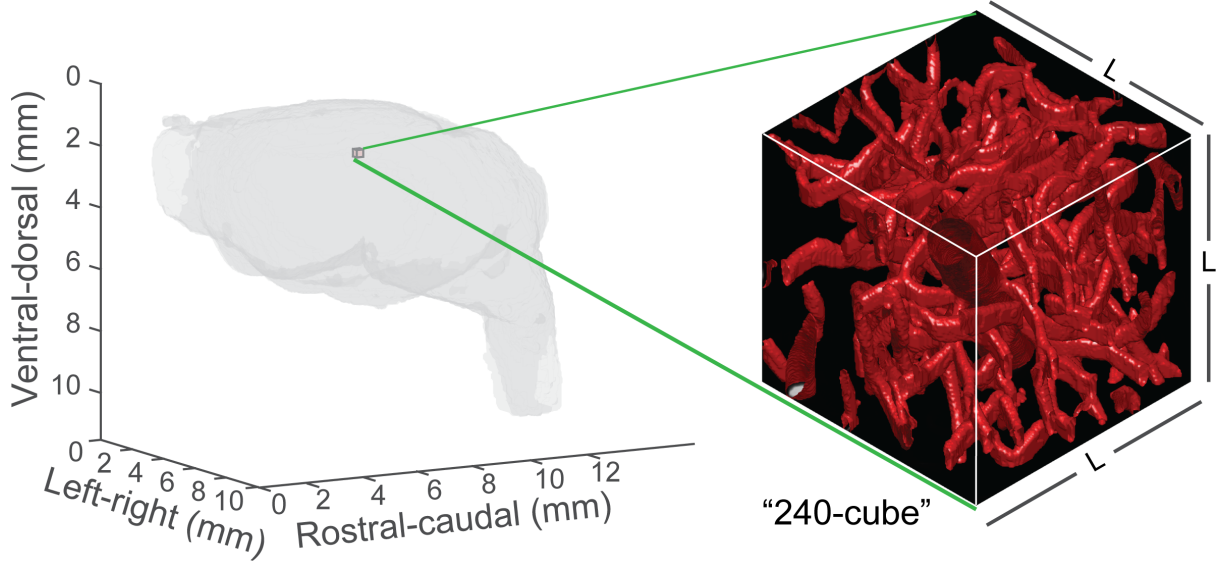


Figure 3.1. Visualization of Mouse Brain with Vessel Mask in a 240-cube. Left: Mask of the whole mouse brain. Right: Vessel segmentation in one of the $L^3 = (240 \mu m)^3$ image volumes denoted as "240-cube".

large dataset for vascular connectome reconstruction, we downsampled the images to $1 \mu m$ isotropic voxel size and decompose it into $(240 \mu m)^3$ image cubes, each cube has $32 \mu m$ overlaps with its 6-neighbors (top, bottom, front, back, left, right)(Figure 3.1 Left). Each brain is covered by about 50,000 of the 240-cubes.

The size of the cube and overlap were chosen to optimize for parallel numerical processing. The image segmentation algorithm involves several computationally expensive 3D convolution and matrix factorizations, both were computed on the GPUs. Convolution was computed using the Cross-Correlation Theorem. As a recursive algorithm, the Fast Fourier Transform (FFT) is most efficient when the array size is a power of 2. The array size for FFT is the sum of the image size and the filter size, whose maximum size was chosen based on the scale of microvessels ($10 \mu m$). We determined that an array size of around $(256 \mu m)^3$ was most suitable for parallel computing on our GPUs (with 8 to 11 GB of memory) and a maximum filter size of $16 \mu m$.

3.2 3D vascular image segmentation

The first step is to perform 3D vascular image segmentation to label each voxel in the image as a potential vessel voxel or as background. We chose classical digital image processing techniques, including match-filtering, analyzing the eigenvalues of the local Hessian matrix, adaptive thresholding, and hysteresis thresholding, mainly due to their robustness and efficiency. Our limitations in computational resources and evolving sample preparation and image protocols were also the reason we did not develop more sophisticated algorithms like deep neural network[16] for 3D image segmentation.

For each 240-cube, a $3 \times 3 \times 3$ median filter was applied to remove shot noise before background noise level estimation, contrast stretching, and intensity normalization. The resulting image cube I was down-sampled 8 times by max-pooling to calculate the local intensity maximum I_{max} and the local intensity threshold I_{th} was estimated by

$$I_{th} = \max \left\{ 0.35I_{max}, I_{bg} \right\}, \quad (3.1)$$

where I_{bg} was estimated by the mean and standard deviation of the estimated background voxel intensity. I_{th} was smoothed by a 3D Gaussian filter, up-sampled, and used to produce an intensity-based vessel mask M_{int} . We assumed the original image was not saturated and therefore voxels of intensity greater than half of the maximum bit value were also added to the intensity mask M_{int} .

Then, we calculated the maximum response of each image voxel to rod-shaped filters at different orientations[121]. For a rod filter orientated at (θ, ϕ) in spherical coordinate, a 3D filter array was generated by:

$$rod(\mathbf{r}; \theta, \phi) = \mathcal{N} \exp \left[-\mathbf{r}^T \left(\mathbf{R}(\theta, \phi)^T \text{diag}(\sigma_X^{-2}, \sigma_Y^{-2}, \sigma_Z^{-2}) \mathbf{R}(\theta, \phi) \right) \mathbf{r} \right], \quad (3.2)$$

where $\mathbf{r} = (x, y, z)$, $\text{diag}(\cdot)$ was the diagonal matrix and the rotation matrix $\mathbf{R}(\theta, \phi)$ was defined

as:

$$\mathbf{R}(\theta, \phi) = \mathbf{R}_Y(\theta)\mathbf{R}_Z(\phi) = \begin{bmatrix} \cos(\theta) & 0 & \sin(\theta) \\ 0 & 1 & 0 \\ -\sin(\theta) & 0 & \cos(\theta) \end{bmatrix} \begin{bmatrix} \cos(\phi) & \sin(\phi) & 0 \\ -\sin(\phi) & \cos(\phi) & 0 \\ 0 & 0 & 1 \end{bmatrix} \quad (3.3)$$

To approximate a cylinder, σ_Y , and σ_Z were set to be $1 \mu m$ to match the scale of capillaries, while σ_X was set to be three orders of magnitude greater σ_Y . The length of the cylinder was set to be $7 \mu m$. Voxels with values smaller than e^{-1} were zeroed and $\mathcal{N}$ was the normalization factor calculated after zeroing. Since the 3D Gaussian distribution was centrosymmetric, we generated 36 rod filters of different orientations that uniformly cover the upper half of the unit sphere. These filters were convoluted with the image using Fast Fourier Transform (FFT) and the maximum response of the correlation value for each voxel was recorded in an array as the enhanced image I_e .

I_e was used to compute the Frangi vesselness filter [31] at the scale of $\sigma_s \in \{0.5, 1, 2\} \mu m$. After smoothing I_e with a 3-dimensional isotropic Gaussian kernel $G(0, \sigma_s)$ with standard deviation σ_s , the γ -normalized derivatives were computed to form the Hessian matrices. The (μ, ν) element of the Hessian matrix for voxel (i, j, k) was given by:

$$H_{\mu, \nu}(i, j, k) = \sigma_s^\gamma \left[\frac{\partial^2}{\partial \mu \partial \nu} \right] \tilde{I}_e(i, j, k; \sigma_s) \quad (3.4)$$

where $\gamma = 1$ and $\tilde{I}_e(i, j, k; \sigma_s) = G(0, \sigma_s) * I_e$. As a 3×3 real symmetric matrix, the eigenvalues of the Hessian matrix $\lambda_1, \lambda_2, \lambda_3$ were computed using the analytical formula [114] and sorted in ascending order according to their absolute value ($|\lambda_1| \leq |\lambda_2| \leq |\lambda_3|$). The Frangi vesselness response at scale σ_s was:

$$\mathcal{V}_{\sigma_s} = \begin{cases} 0 & , \lambda_2 > 0 \text{ or } \lambda_3 > 0 \\ (1 - e^{-\frac{1}{\alpha} \frac{\lambda_2^2}{\lambda_3^2}}) e^{-\frac{1}{\beta} \frac{\lambda_1^2}{|\lambda_2 \lambda_3|}} (1 - e^{-\frac{\sqrt{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}}{c}}) & , \text{ otherwise} \end{cases} \quad (3.5)$$

The vesselness response was repeated for all the scales and the final vesselness response was the maximum response across multiple scales:

$$\mathcal{V}(i, j, k) = \max_{\sigma_s} \mathcal{V}_{\sigma_s}(i, j, k) \quad (3.6)$$

The vessel mask M_v was produced by thresholding the Frangi vesselness response and combined with the intensity mask M_{int} by a voxel-wise AND operation. The Frangi filter provided a relatively reliable way to recognize the cylindrical objects but failed to detect the branch point due to the more complicated geometry. To improve the connectivity accuracy, adaptive hysteresis thresholding was applied to the region outside of the M_v to add voxels of high local signal-to-noise (SNR) level near the existing mask into M_v . The local SNR was computed using the average and standard deviation of the voxel intensity within a window, which can be efficiently computed using convolution. Finally, small connected components in the segmentation were removed before the vessel mask (Figure 3.1 Right) in a 240-cube was saved. Leveraging the sparsity of vasculature in most of the brain region, the vessel mask is stored as the vessel voxel coordinates along with other metadata specifying the size and position of the volume in the integrated $1\mu m$ resolution space. For efficient downstream reconstruction, the 3-dimensional coordinates of the voxels in the mask were converted to their linear indices in the 240-cube.

3.3 Skeletonization

Based on the vessel mask, we extracted the skeleton (centerline) of the vascular network using a topology-preserving morphological thinning algorithm[68]. Given a 3D binary array, this algorithm iteratively removes a "removable simple point" from the foreground. The "removable simple point" was a voxel that (i) is not an endpoint (with only 1 26-neighbor) and (ii) its removal does not change the topology (measured by the Euler characteristic) of the existing foreground connected component that it belongs to.

Ideally, this thinning algorithm should operate on the binary mask of whole brain vascular

to generate a continuous skeleton. However, whole mouse brain vascular network at $1\ \mu m$ resolution still spans a space of about 1 trillion voxels (10^{12}), which is beyond the capacity of our workstation. Consequently, we developed a skeletonization procedure that computed the vessel centerline in partially overlapping $(1072\mu m)^3$ cubes (denoted as 1072-cubes) and then merged the skeletons from all these volumes for subsequent graph construction. As the thinning process depends on examining the neighbors of foreground voxels, volume boundary inevitably biases the resulting skeleton. The maximum distance between the biased skeleton voxel and the edge of the volume is set by the distance between the voxel and the real boundary of the object. Therefore, we constructed 1072-cubes by merging $5 \times 5 \times 5$ 240-cubes (Figure 3.2A), each 1072-cube has $240\ \mu m$ overlap with its 6-neighbors. For each 1072-cube, vessel masks in its 125 240-cubes were stitched and refined by morphological operations to fill the potential small holes, skeletonized, divided back to 240-cubes, and saved. 240-cubes on the surface of a 1072-cube might have skeleton coordinates from the skeletonization result of adjacent 1072-cubes. The skeleton voxel position was updated based on the distance between the 240-cube and the edge of the corresponding 1072-cube.

Note that this algorithm only produced a skeleton that approximates the centerline. To refine the position of the skeleton voxels for radius estimation, we assumed that microvessels have the brightest intensity along the centerline and moved the skeleton voxels to their brighter neighbors if the adjustment did not alter the topology of the skeleton. For each skeleton voxel, we searched for brighter background voxels in its 26-neighboring, among which we selected the brightest one that (1) was a "removable simple point" in the original configuration, and (2) made the original skeleton voxel a "removable simple point" in its 26-neighbors if it was added to the skeleton (blue green voxels, Figure 3.2B). This "centering" procedure was carried out iteratively until no skeleton voxel could be moved. Voxels that became "removable simple points" were removed along the way of centering.

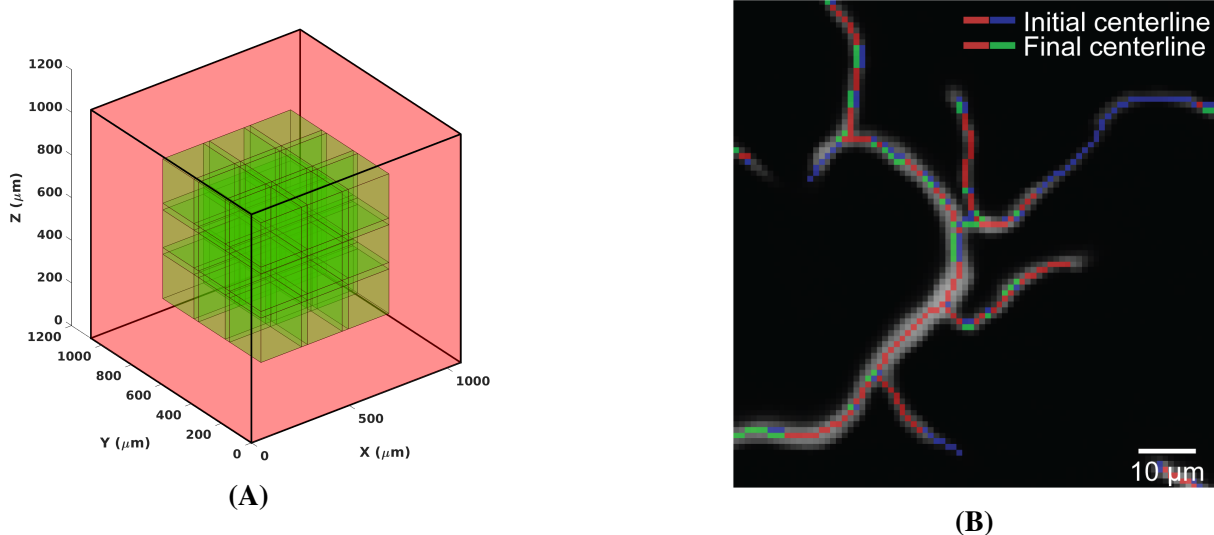


Figure 3.2. Illustration of the skeletonization process. (A) Illustration of a 1072-cube (red) with 27 internal 240-cubes (green). (B) Illustration of vessel centerline adjustment. Centerline voxels were moved to a neighboring position with higher intensity if the adjustment preserved the topology. Unmoved voxel: red. Moved voxel before adjustment: blue. Moved voxel after adjustment: green.

3.4 Spatial graph construction

Our goal is to construct the spatial graph representation of the whole brain vascular network. This graph contains the position and radius of all the vessels and the connectivity between all the segments. The spatial graph construction process started by classifying skeleton voxels into endpoint, link, and node voxels, according to the number of skeleton voxels in their 26 neighbors. Voxels with more than 2 neighbors were used to construct a mask, from which the connected components (CC) formed the nodes in the graph. The remaining voxels were used to construct another mask, where the connected components formed the edge of the graph. For each edge connected component, the linear indices of the positions of all its skeleton voxels are ordered from one end of the segment to another end. To enable a rapid reconstruction of the graph on the whole-brain scale, sparse matrices were used for searching the connected components. The radius associated with each skeleton voxel was assigned as the distance from the voxel to the nearest boundary between the vessel and void using 3D Euclidean Distance Transform (DT).

This forms a primitive vascular graph. This algorithm enables the construction of the whole mouse brain vascular connectome at 1 μm resolution on a desktop workstation in about 1 hour.

A MATLAB structure was designed to store the position and radius of every centerline voxel in the node and edge connected components, as well as the connectivity between nodes and edges. The MATLAB structure is organized as follows:

- **num**: structure with the following fields:
 - **mask_size**: 1 x 3 vector, size of the 3D array that holds the vascular network. The dimensions are in MATLAB subscript order and can be directly used for converting the linear indices of the voxels into subscripts.
 - **mask_size**: 1 x 3 vector, size of the 3D array that holds the vascular network. The dimensions are in MATLAB subscript order and can be directly used for converting the linear indices of the voxels into subscripts.
 - **mask_size_pad**: 1 x 3 vector, equals **mask_size** + 2.
 - **block_voxel**: scalar, number of voxels in the 3D array.
 - **block_voxel_pad**: scalar, number of voxels in the padded 3D array.
 - **neighbor_add_pad**: 26 x 1 vector, linear indices difference between a voxel and its 26 (3 x 3 x 3 - 1) neighboring voxels in the 3D array.
 - **neighbor_add_pad**: 26 x 1 vector, linear indices difference between a voxel and its 26 (3 x 3 x 3 - 1) neighboring voxels in the padded 3D array.
 - **skeleton_voxel**: scalar, number of vessel skeleton voxels.
- **link**: structure with the following fields:
 - **num_cc**: scalar, number of link (vessel segment) connected components in the graph.
 - **cc_ind**: cell array, each cell contains the linear indices of the centerline voxels in the link, ordered from one end of the segment to another end of the segment. The link

- connected components are labeled from 1 to **num_cc**.
- **pos_ind**: column vector, generated by concatenating **link.cc_ind**.
 - **num_voxel**: number of link voxels in the graph.
 - **num_voxel_per_cc**: number of voxels in each link connected component, i.e. number of elements in each cell of **cc_ind**.
 - **label**: column vector, link labels of the link voxels.
 - **map_ind_2_label**: sparse column vector, map the linear indices of link voxels to their link connected component labels.
 - **connected_node_label**: **num_cc**-by-2 array, labels of the connected nodes at the two ends (may not in the same order as the indices in **cc_ind**). 0 represents unconnected endpoints.
 - **num_node**: column vector, number of connected nodes of each link.
- **edge**: structure with the following fields:
 - **num_cc**, **num_voxel**, **num_voxel_per_cc**, **label**, **map_ind_2_label**, **pos_ind** are defined the same as the ones in **link** for node connected component.
 - **cc_ind**: same as **link.cc_ind**, but the linear indices in each cell are not ordered.
 - **connected_link_label**: cell array, each cell contains the label of the link connected component that the node is connected to.
 - **isopoint**: structure with the following fields:
 - **pos_ind** and **num_voxel** are defined the same as the ones in **link** for isolated centerline voxels.
 - **isoloop**: structure with the following fields:

- **cc_ind**, **num_cc**, **pos_ind** are defined the same as the ones in link for isolated loop connected components.
- **endpoints**: structure with the following fields:
 - **pos_ind**: column vector, linear indices of the link voxels with only one 26-neighbor centerline voxel
 - **link_label**: column vector, the label of the link connected component that the endpoint voxel belongs to.
 - **num_voxel**: scalar, number of endpoint voxels.
 - **map_ind_2_label**: sparse column vector, map the linear indices of the endpoint voxels to their link connected component labels.
- **radius**: sparse column vector, map the linear indices of the centerline voxels to their radii.

3.5 Learning-based graph refinement

Though the algorithm preserved topological characteristics of the vessel mask, the thinning process usually generates many short (a few voxels long) skeleton segments with one free endpoint(Figure 3.3A). Moreover, the primitive vessel graph contained incorrect connectivity information that was inherited from the segmentation error. On the one hand, vessels that were a few micrometers apart might be merged due to the relatively large spread of the point spread function (PSF) in the axial direction(Figure 3.3B). On the other hand, capillaries near arteries or veins might be disconnected due to stronger background levels and less contrast. Moreover, a very small fraction of capillaries contained stalled blood cells or were not completely filled(Figure 3.3C, Figure 3.3D), with additional complications from imaging and image stitching artifacts.

To reduce the connectivity error, We developed an automatic graph refinement procedure that involved the removal of isolated voxels, short segments with one or two unconnected

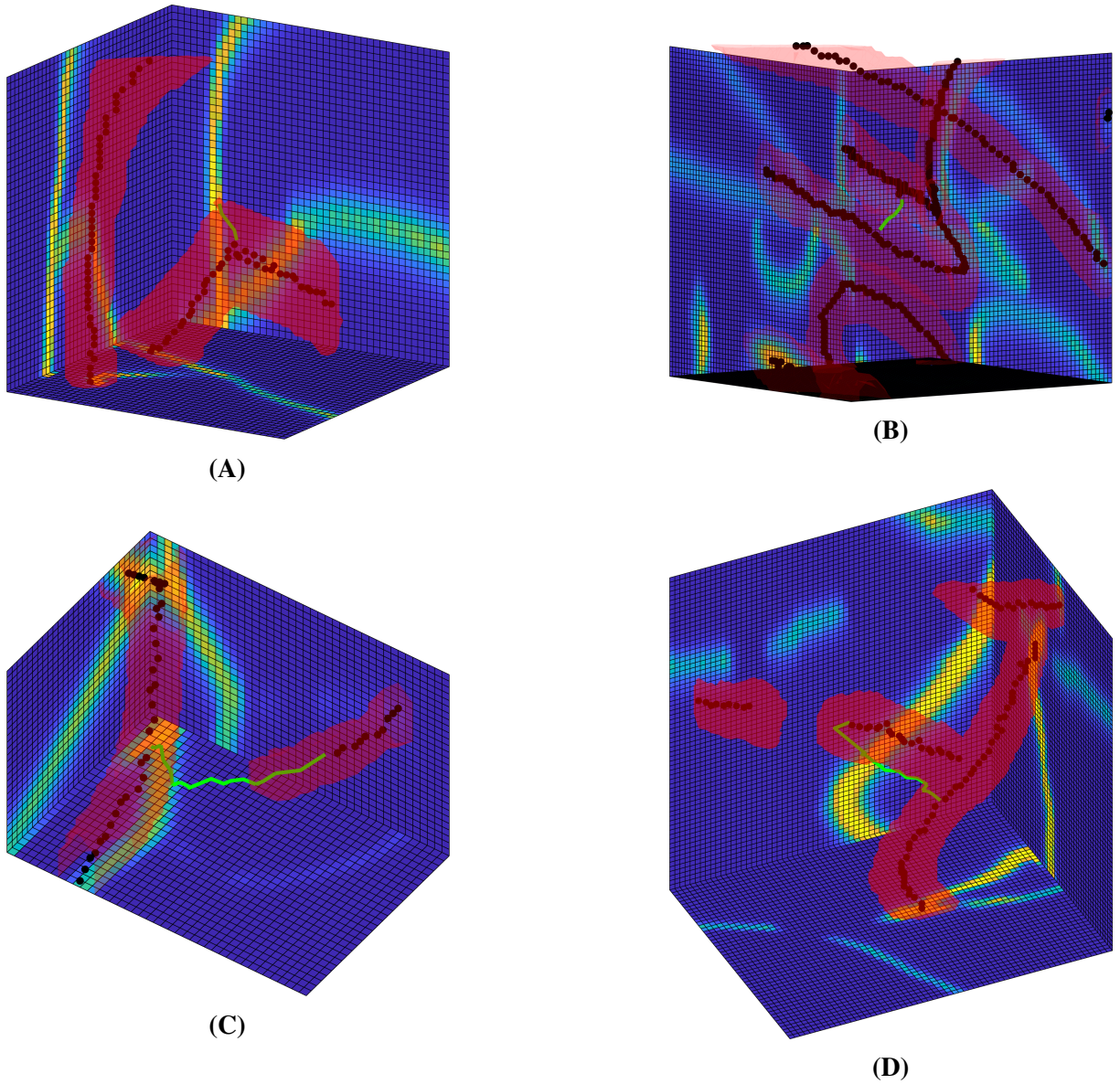


Figure 3.3. Illustration of graph refinement target. Illustration of candidate vessel segments for the graph refinement procedure. The reconstructed vessels are in red, the neighboring centerlines are in black, and the segments to be classified are in green. Note the presence of the maximum projections along the surfaces of the cube. (A) A vessel segment with one free endpoint that needs to be removed. (B) A false positive connection that results from relatively low axial imaging resolution and proximity of vessels. (C) A correctly proposed linker that needs to be added to the centerline. (D) An incorrectly proposed linker should not be added.

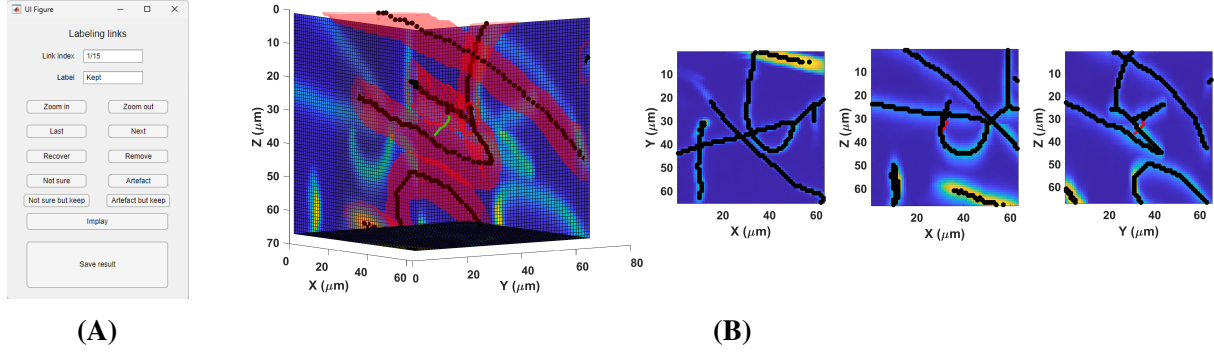


Figure 3.4. Graph refinement annotation GUI. (A) Annotation graphical user interface control panel. (B) Illustration of candidate vessel segments for labeling. The reconstructed vessels are in red, the neighboring centerlines are in black, and the segments to be classified are in green. Note the presence of the maximum projections along the surfaces of the cube. A false positive connection that results from relatively low axial imaging resolution and proximity of vessels.

endpoints (Figure 3.3A), and false positive connections (Figure 3.3B). It further involved the addition of a set of voxels to bridge free-ended segments and form a contiguous centerline [57] (Figure 3.3C, Figure 3.3D). The main component of this procedure is random forest classifiers. We developed graphical user interface (GUI) to annotate thousands of links across different brain regions efficiently (Figure 3.4). Thousands of link CCs from a few 1072-cubes in different regions of the brain were labeled. We then designed tens of intensity, geometrical, and topological features for the labeled segments. These segments were randomly split into 80% and 20% for training and validation, respectively. The resulting classifiers were used for automatically refining the vessel graph in the whole brain. All classifiers achieved more than 95% accuracy with respect to manual annotation. As a measure of the improved structural accuracy, 49% of the 240-cubes that did not include an outer brain surface also did not contain any vessel segments with an endpoint (Figure 3.5).

3.5.1 Recursive graph pruning

First, short free-ended links and loops consisting of one or two links were deleted. The short free-ended links were mainly artifacts of the skeletonization algorithm, while short loops were the result of stitching artifacts and noise. Among all the possible configurations of loops, a



Figure 3.5. Graph refinement classifier confusion matrices. (A) Classifier for removing links with 1 endpoint. (B) Classifier for removing false positive links due to lower axial resolution. (C) Classifier for removing false linkers. (D) Quantifying fraction of vessels with endpoints in all the 240-cubes that are completely inside the brain.

”self-loop” is a link that connects a node back to itself, while a ”bi-link loop” has a pair of links connecting a pair of nodes. Self-loops are not biologically plausible, though ”apparent” long self-loops could appear when one of the nodes of bi-link loops happens to be on the edge of the 1072-cube. Short bi-link loops were found to be mainly artifacts under visual inspection. As deleting a link changed the identity of the connected links, the graph needed to be iteratively updated during deletion, as described below:

Algorithm 1: Delete short free-ended links and short loops

```
needRefinementQ = true;

while needRefinementQ do
    Delete free-ended link of length  $\leq 2 \mu m$ ;
    Find links that connect a node to itself ("self-loop") and delete the ones of length
         $\leq 30 \mu m$ ;
    Find loops that consist of two links and at least one of the links of length
         $\leq 15 \mu m$ . For each of these loops, delete its link with a lower average
        fluorescence intensity;
    Update the graph;
    if no link deleted then
        | needRefinementQ = false;
    end
end
```

3.5.2 Classifying links with single unconnected endpoint

The remaining free-ended links could be artifacts or incompletely labeled vessels. For links with one unconnected endpoint, a random forest classifier was trained to further identify segments for removal using the following features:

- Segment length
- Maximum, minimum, mean, standard deviation, and coefficient of variance of the skeleton voxel radii
- Median, coefficient of variance, and SNR level of the skeleton voxel intensity
- Intensity difference between the two voxels at the end of the segment
- Ratio between maximum skeleton voxel radius and segment length

- Distance to the nearest unconnected endpoint voxel
- Inner product between the segment tangent vector and the nearest unconnected endpoint tangent vector
- Number of skeleton voxels in the segment of the nearest unconnected endpoint

3.5.3 Classifying short links with low SNR level

The inter-capillary distance could be only a few micrometers, which is comparable to the axial optical resolution. These nearby vessels might be incorrectly connected by the segmentation algorithm. We trained classifiers to reduce this type of false positive connection. First, we selected vessel segments with the following criteria as candidates:

- Minimum skeleton voxel intensity is lower than a threshold (depends on the imaging system and image intensity histogram)
- Segment length $< 25 \mu m$
- Ratio between the sum of endpoints radii and segment end-to-end distance > 0.15

Another random forest classifier was trained to identify the false positive connections among the candidates using the following features:

- Segment length and end-to-end distance
- Maximum, minimum, median, standard deviation, and coefficient of variance of the skeleton voxel radii
- Minimum, Mean, median, standard deviation, and coefficient of variance of the skeleton voxel intensity
- Relative position of the minimum intensity voxel along the segment skeleton, and its normalized distance to the middle

- Intensity of the middle skeleton voxel, intensity difference between the middle skeleton and the minimum intensity of the skeleton voxels
- Ratio between the sum of endpoints radii and segment end-to-end distance
- Ratio between the difference in endpoints radii and the segment length
- Ratio between the middle intensity and the maximum intensity of the skeleton voxel
- Ratio between the middle intensity and the maximum intensity at the two ends of the segment
- SNR level of the surrounding vessel mask, segment skeleton, and the middle skeleton voxel
- Difference between average endpoint intensity and the middle skeleton voxel intensity, normalized by the average endpoint intensity
- The z-component of the segment end-to-end unit vector

3.5.4 Purposing and selecting linkers

For the remaining free-ended links, a modified version of the threshold relaxation algorithm [57] was used to propose the possible voxel lists (linkers) that connect the unconnected endpoints to their surrounding skeleton. First, the local volume of vessel image, existing vessel mask, and skeleton were cropped around the unconnected endpoint. The cropped vessel image was then thresholded by iteratively decreasing the intensity threshold until getting a CC that contained both the unconnected endpoint and voxels in the original vessel mask that are not connected to the unconnected endpoint in the cropped volume. This CC defined the search space for finding the "linker" to bridge the endpoint to its neighboring skeleton. Voxels in this search space were used to construct an undirected graph, where each node had intensity-weighted edges connecting to their 6-neighbors in the CC. Dijkstra's shortest path algorithm was applied to obtain an ordered set of voxel coordinates (denoted as a linker) that maximizes the SNR level.

For each linker, the following features were computed, and the third random forest classifier was trained to further select the linkers to be added to the existing skeleton.

- Original length of the link with the endpoint
- Number of links in the cropped volume
- Number of adjacent links of the linker
- Linker length, end-to-end distance, straightness, end-to-end vectors of the linker
- Ratio between the length of the linker and the link with the endpoint
- Mean, standard deviation, and signal-to-noise level of the linker voxel intensity
- Mean, standard deviation, and signal-to-noise level of the linker voxel intensity outside the original vessel mask
- Linker radius (derived from the radii of the skeleton voxels near the two ends of the linker)
- Mean, median, standard deviation, and SNR level of the voxel intensity in the reconstructed mask of the linker
- Mean and standard deviation of the intensity of the background voxels near the linker mask
- Mean and standard deviation of the ratio between linker-skeleton distance and linker-endpoint distance
- Inner product between the endpoint tangent vector and the linker end-to-end vector
- Number of unconnected endpoints in the link
- Number of times the derivative of the linker voxel - skeleton distance change sign

For a link with one unconnected endpoint, the endpoint voxel might be converted to link voxel if the linker was added, or remain an endpoint voxel if the linker was rejected by the classifier. For a link with two unconnected endpoints, if the linkers for both endpoints were rejected, the link was deleted directly. Otherwise, the link might become part of a new link with 0, 1, or 2 unconnected endpoints. The remaining links with endpoint(s) could be further refined in the next iteration. The entire graph refinement process was repeated 3 times for each 1072-cubes. In summary, the graph refinement process could be abstracted as below:

Algorithm 2: Graph refinement

```

i = 0; needRefineQ = true;
while  $i < 3$  & needRefineQ do
    i = i + 1;
    Delete short free-ended links and short loops;
    Find, quantify, and classify links with a single endpoint to delete. Update graph;
    Find, quantify, and classify short links with low SNR to delete. Update graph;
    Find, quantify, and classify linkers to add. Update graph;
    Delete unmodified links with two unconnected endpoints;
    Delete short free-ended links and short loops;
    if graph is not modified then
        | needRefineQ = false
    end
end

```

3.6 Super-resolution vessel radius estimation

Vessel radius is a crucial geometric parameter of vessel structure that determines flow dynamics. Blood flow in the capillary is characterized by a low Reynolds number[105]. To the first-order approximation, laminar blood flow follows the Hagen-Poiseuille equation, where 10% uncertainty in radius estimation leads to 40% uncertainty in flow resistance. Therefore,

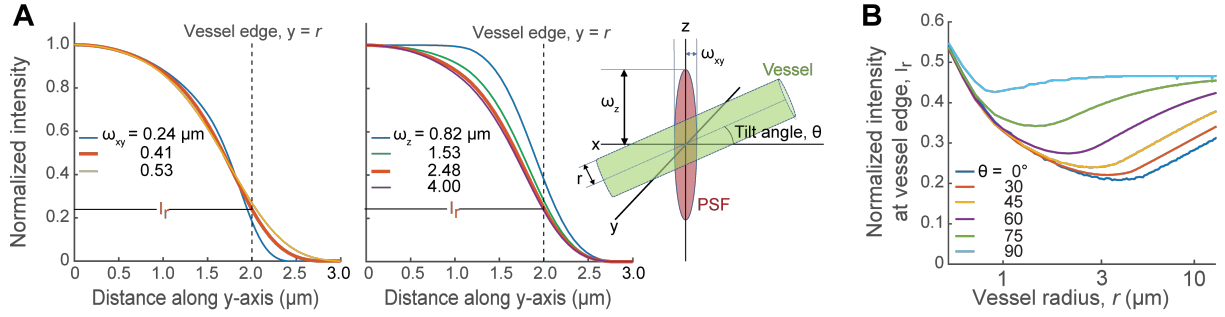


Figure 3.6. Model-based vessel edge determination. (A) Calculated normalized horizontal radial light intensity profile of a vessel. The profile is along the y -axis of the vessel in the insert, with $\theta = 0^\circ$ and $r = 2.0 \mu\text{m}$, for a PSF of the beam with different ω_{xy} ($\omega_z = 3.4 \mu\text{m}$; left) and different ω_z ($\omega_{xy} = 0.41 \mu\text{m}$; right), respectively. (B) Normalized horizontal vessel edge intensity for vessels of different radius and orientation ($\omega_{xy} = 0.44 \mu\text{m}$ and $\omega_z = 3.4 \mu\text{m}$).

high-precision estimation of the capillary radius is crucial for constructing a vascular graph that is suitable for quantitative blood flow simulation.

Without interpolating the radial intensity profile, the lower bound of radius estimation uncertainty came from discretized sampling, which for $2 \mu\text{m}$ radius vessel, is about 3%, 10% and more than 20% for $0.25 \mu\text{m}$, $1 \mu\text{m}$ and $1.6 \mu\text{m}$ lateral resolution, respectively. Moreover, the vessel edge under the microscope was blurred by the point spread function (PSF) of the imaging system. Therefore, high-precision radius measurement required both sub-micrometer voxel resolution imaging and super-optical-resolution localization of vessel edge.

We exploit the sparseness of vessels and developed an algorithm to joint-estimate PSF and vessel radius from $0.25 \mu\text{m} \times 0.25 \mu\text{m} \times 1 \mu\text{m}$ resolution images (Figure 3.7).

3.6.1 Vessel image profile simulation

We first formed a look-up table of the expected intensity profile, denoted:

$$I = I(x, y, z; r, \theta, \omega_{xy}, \omega_z), \quad (3.7)$$

for a vessel in terms of four parameters: the vessel radius, denoted r , the elevation angle with respect to the focal plane, denoted θ , and the three-dimensional Gaussian point spread function

(PSF). The vessel was locally approximated as a cylinder along with uniform lumen fluorescence density. The elevation angle of the vessel is readily determined from the skeletonization procedure and used without further modification. The PSF is characterized by lateral and axial half-widths-at-half-maximum intensity, denoted ω_{xy} and ω_z , respectively (insert, Figure 3.6A). The 3D theoretical vessel images were computed as the convolution between the cylinder and PSF. The simulation voxel size was chosen to be less than 20% of the smallest relevant scale (r , σ_{xy} , and σ_z). For each set of parameters, lateral radial (along y-direction) and axial radial (along z-direction) intensity profile ($I_{xy}(y|r, \theta, \sigma_{xy}, \sigma_z)$ and $I_z(z|r, \theta, \sigma_{xy}, \sigma_z)$) were recorded and the normalized edge intensity in the radial direction on horizontal plane was defined as:

$$I_{th}(r, \theta, \omega_{xy}, \omega_z) = \frac{I_{xy}(r|r, \theta, \omega_{xy}, \omega_z)}{I_{xy}(0|r, \theta, \omega_{xy}, \omega_z)}. \quad (3.8)$$

The intensity profiles were computed for series of radius ($r \in [0.5, 15] \mu m$), elevation angle ($\theta \in [0, \pi/2]$) and PSF size ($FWHM_{xy} \in [0.45, 2] \mu m$ and $FWHM_z \in [1.33, 12] \mu m$). The intensity at the edge of a vessel, denoted I_r at point $(x, y, z) = (0, r, 0)$ in the insert of Figure 3.6A, decreases as both lateral and axial resolution decrease and depends nonlinearly on vessel radius and elevation angle (Figure 3.6). The intensity at an edge is least sensitive to changes in radius when the vessel lies in the axial direction and is highly sensitive to changes for vessels that lie in the focal plane (Figure 3.6B). Note that with realistic vessel and PSF size, the normalized edge intensity is always smaller than 0.5, and therefore thresholding vessel images with a normalized intensity threshold of 0.5 systematically underestimates the radius.

3.6.2 Radius estimation with a known PSF

If the actual PSF is known, vessel radius estimation can be iteratively refined (Figure 3.7). For a skeleton voxel in $1 \mu m$ resolution reconstruction, a small image volume around its corresponding point in the full resolution images was cropped (Figure 3.7 Panel 1) and normalized. Its neighboring skeleton voxels were used to compute the elevation angle θ of the vessel segment

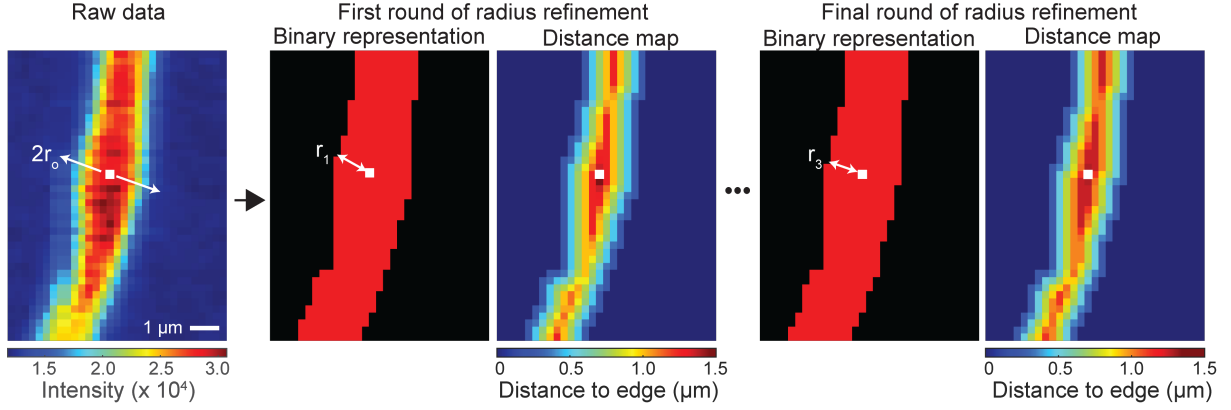


Figure 3.7. Super-resolution vessel radius estimation steps. Determination of the radius associated with a voxel that lies at the centerline (white square) of the vessel. Iterative progression from raw data to final super-resolution estimate of the radius. The raw data has the centerline tilted at $\theta = 6^\circ$ and an initial estimate $r_0 = 2 \mu\text{m}$. Then initial super-resolution estimate of r_1 is determined by thresholding the intensity profile of the raw data at the value of I_{r_0} (panel C) which leads to the new estimate, read from the distance map, of $r_1 = 1.46 \mu\text{m}$. This process is repeated until the radius converges to a final value of $1.35 \mu\text{m}$.

with respect to the optical axis. With an initial radius r_0 estimated at $1 \mu\text{m}$ resolution, normalized edge intensity I_{r_0} was computed, for thresholding the cropped image volume. The new estimate of radius, r_1 , was computed as the local maximum of the 2D distance transform (“distance map”) of the resulting binary vessel mask (Figure 3.7 Panel 2, 3). For a vessel of radius $2 \mu\text{m}$ on the lateral plan, if initially the radius was overestimated by 70%, the estimated radius after 1 iteration would be overestimated by less than 3% (Figure 3.8A). This process was continued until the estimate of the radius converged. Typically this process converged by three iterations (r_3 in Figure 3.7). The algorithm is summarized in algorithm 3.

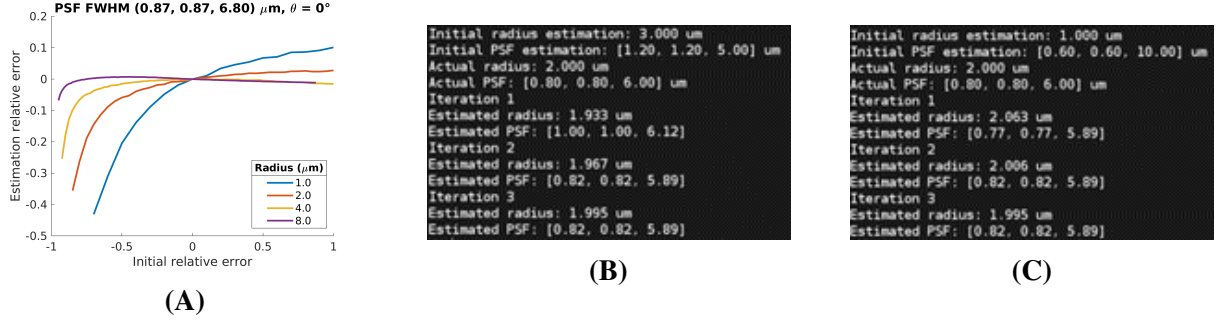


Figure 3.8. Convergence of radius-PSF joint estimation. (A) Relative error in vessel radius before and after 1 step of the estimation procedure. (B) An example of the convergences of radius-PSF joint estimation with initially over-estimated radius and PSF size. (C) An example of the convergences of radius-PSF joint estimation with initially under-estimated radius and PSF size.

Algorithm 3: Radius estimation

```

 $r_0 = -1$ ;  $n = 1$ ;  $N = 6$ ;
Initialize radius estimate  $r_e$  from vessel segmentation;
Calculate skeleton voxel position  $\mathbf{x}_s$  in  $0.25 \mu\text{m}$  resolution image stack;
Crop  $0.25 \mu\text{m}$  resolution image stack around  $\mathbf{x}_s$  according to  $r_e$ , denote as  $I(\mathbf{x})$ ;
Locally search for the intensity maximum  $I_{max}$  around  $\mathbf{x}_s$ ;
Estimate background light intensity level  $I_{bg}$ ;
Normalize light intensity:  $I_n(\mathbf{x}) = \frac{I(\mathbf{x}) - I_{bg}}{I_{max} - I_{bg}}$ ;
while  $r_0 \neq r_e$  &  $n \leq N$  do
     $r_0 = r_e$ ,  $n = n + 1$ ;
    Use  $r_e$  and  $\theta$  to calculate normalized edge intensity  $I_{th}(r_e, \theta, \sigma_{xy}, \sigma_z)$ ;
    Generate local vessel segmentation  $M(\mathbf{x}) = I_n(\mathbf{x}) \geq I_{th}$ ;
    Apply morphological close to  $M(\mathbf{x})$ ;
    Compute 2D distance transform  $D(\mathbf{x})$  for each horizontal plane of  $M(\mathbf{x})$ ;
     $r_e = \text{Local maximum of } D(\mathbf{x}) \text{ around } \mathbf{x}_s$ ;
end

```

3.6.3 Radius - PSF joint estimation

The aforementioned radius estimation method relied on precise measurement of the PSF. We next refined our estimate of the PSF using the refined radii associated with the centerline points. For each centerline voxel, the new estimation of PSF parameters was determined by maximizing the correlation between the measured and calculated radial intensity profiles along

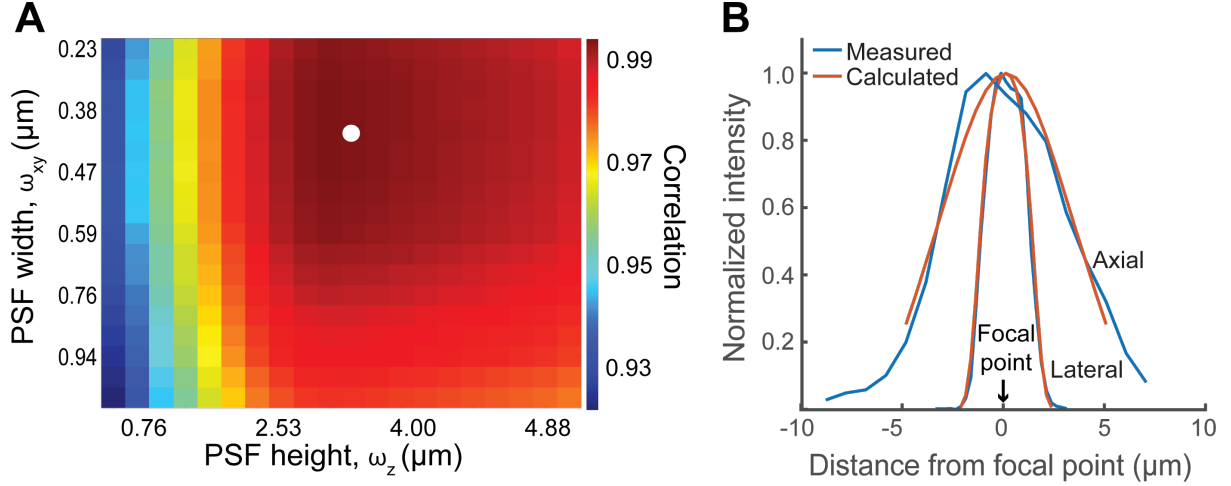


Figure 3.9. Joint PSF-radius estimation. (A) Estimate PSF by maximizing the weighted-average normalized correlation between the simulated intensity profiles and the actual profile. White dot shown the optimal PSF parameters ($\omega_{xy} = 0.41 \mu\text{m}$, $\omega_z = 3.4 \mu\text{m}$). (B) Measured (blue) and simulated (red) lateral and axial intensity for the optimum PSF parameter found in panel D; $r = 1.6 \mu\text{m}$. The weighted average normalized correlation is 0.995.

horizontal and axial directions, weighted by orientation (Figure 3.9A). The newly refined PSF was then used to update the look-up table for the $I(x, y; r, \theta, \omega_{xy}, \omega_z)$ (Figure 3.6B) and the radius of the vessel centerline was recalculated (Figure 2A). This process of nested iteration converged in two to three cycles and yielded a final estimate of the radius associated with each voxel on the centerline (Figure 3.8BC).

For each brain, we estimated PSF near tens of thousands of skeleton voxels and found relatively stable PSF parameters in different brain regions under our imaging condition. For example, for one of the brains (*ML20180815*), the average lateral and axial FWHM measured in neocortex, hippocampus, brainstem, cerebellum and midbrain were $0.87 \pm 0.04 \mu\text{m}$ and $6.8 \pm 0.3 \mu\text{m}$ respectively (Figure 3.10). In principle, the radius-PSF joint estimation could be applied to the whole brain, but due to the tight distribution of averaged PSF across brain regions and to reduce computational time, we used the averaged PSF for whole-brain radius estimation. The algorithm is summarized in algorithm 4.

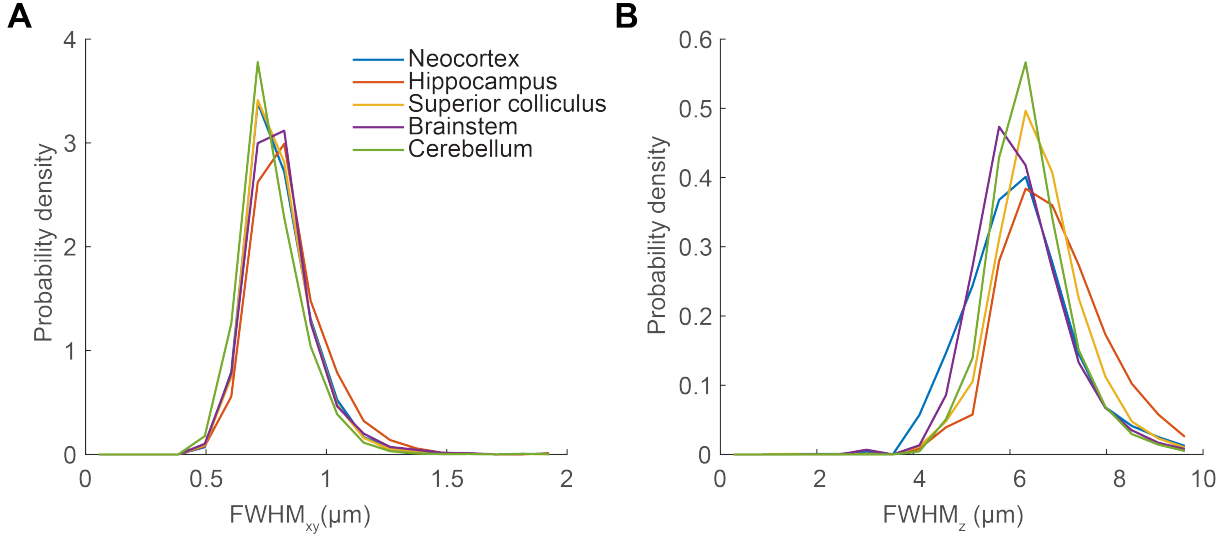


Figure 3.10. Distribution of Point Spread Function in brain tissue. (A) Lateral full-width-at-half-maximum (FWHM) of the PSF estimated in five brain regions. (B) Axial FWHM of the PSF in five brain regions. Number of estimations in both panels: neocortex 9,944; hippocampus 2,368; superior colliculus 5,051; brainstem 4,537; cerebellum 5,268.

Algorithm 4: PSF-Radius Joint Estimation

$\mathcal{L}_{\max} = -1$; $\mathcal{L}_0 = -2$; $n = 0$; $N = 6$;
 Initialize σ_{xy} and σ_z by rough estimation from image;
 Initialize r_e from vessel segmentation;
while $\mathcal{L}_0 < \mathcal{L}_{\max}$ & $n < N$ **do**
 $n = n + 1$, $\mathcal{L}_0 = \mathcal{L}_{\max}$;
 Calculate skeleton voxel position $\mathbf{x}_s = (x_s, y_s, z_s)$ in $0.25 \mu\text{m}$ resolution image stack; Update r_e using Algorithm 3;
 Get axial intensity profile at : $i_z(z|x_s, y_s)$;
 Get lateral radial intensity profile $i_{xy}(\hat{r}|\phi, z_s)$, where ϕ is the azimuth angle of the vessel radial direction, and $\hat{r} = \sqrt{(x - x_s)^2 + (y - y_s)^2}$;
 For all the simulated PSF parameter sets $\{\Sigma_{xy}, \Sigma_z\}$, calculate the angle-weighted normalized cross-correlation $C(\cdot, \cdot)$ between measured light intensity i and simulated light intensity I :

$$\mathcal{L}(\Sigma_{xy}, \Sigma_z) = \frac{1}{1 + \cos(\theta)} \left\{ C(i_{xy}(\hat{r}|\phi, z_s), I_{xy}(\hat{r}|r_e, \theta, \Sigma_{xy}, \Sigma_z)) + \right.$$

$$\left. \cos(\theta) C(i_z(z|x_s, y_s), I_z(z|r_e, \theta, \Sigma_{xy}, \Sigma_z)) \right\};$$

 Update PSF estimation by maximizing $\mathcal{L}$:
 $\{\mathcal{L}_{\max}, \sigma_{xy}, \sigma_z\} = \arg \max_{\Sigma_{xy}, \Sigma_z} \mathcal{L}(\Sigma_{xy}, \Sigma_z)$;

end

3.7 Deformation quantification, calibration and reconstruction inspection

3.7.1 Microvessel radius calibration

We now turn to systematic errors in the estimation of the vessel radii in the living versus gel-perfused brain. We collected data from volumes of microvessels in the parietal cortex using adaptive optics two-photon microscopy to image vessels in the living animal with the blood plasma filled with fluorescein-dextran and, in the same animal, after perfusion with a fluorescein-gel before the brain was extracted. The PSF has $0.2\ \mu\text{m}$ lateral resolution [72]. We iteratively estimated the *in vivo* vessel radius using the same algorithm as for the gel-perfused brains. By registering the post-perfusion vessel skeleton voxels to the *in vivo* skeleton, we measured the change in radius at 102,284 positions (6 mice) to compute the radius calibration curve (Figure 3.11). Vessels with radii less than $\sim 5\ \mu\text{m}$ shrunk after perfusion. We computed a calibration curve and applied it to convert the post-perfusion vessel radii to their inferred *in vivo* values. Of interest, the minimum *in vivo* vessel radius is $1.9 \pm 0.3\ \mu\text{m}$ (mean $\pm$ SD).

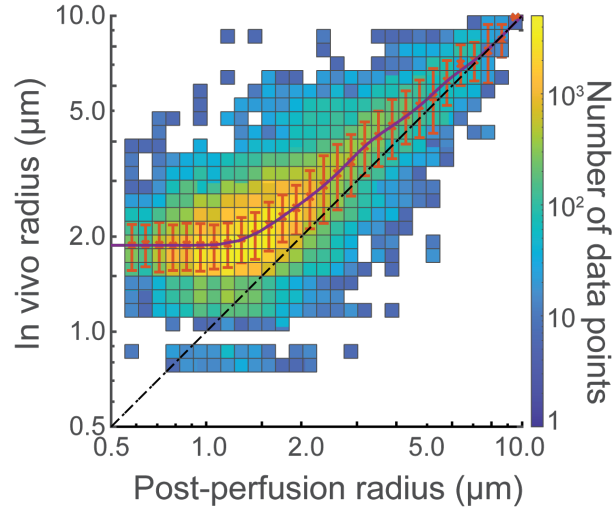


Figure 3.11. *In vivo* - post perfusion radius calibration curve. Joint distribution of *in vivo* vessel radius versus post-perfusion vessel radius of matched vessel centerline voxels from 6 mice. The solid curve is the median of *in vivo* radius binned according to post-perfusion radius. The error bar shows middle quartiles.

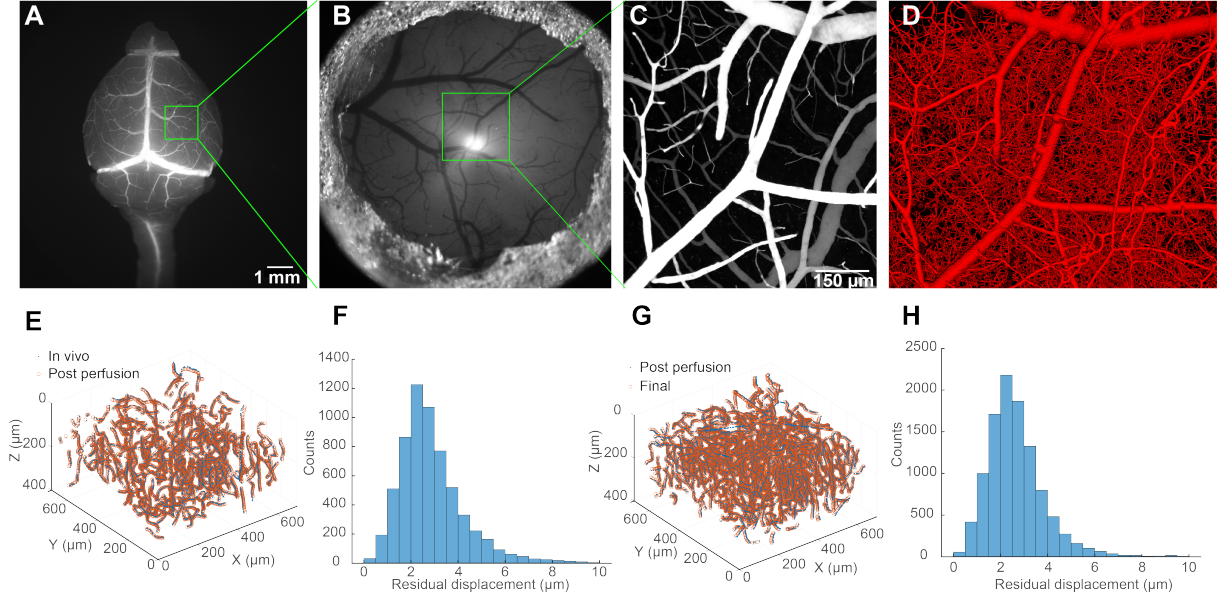


Figure 3.12. Quantify sample deformation. (A) Overview of Brain2 with fluorescent gel-filled vessels. (B) Overview of cerebral surface vessels through a cranial window for *in vivo* and post-perfusion imaging. (C) Max intensity projection of the surface vessels in *in vivo* image. (D) Vessel reconstruction from whole brain imaging. (E) Aligning post-perfusion vessel reconstruction centerline voxels to the *in vivo* reconstruction (Similarity transformation, scaling factor 1.0003). (F) Histogram of residual displacement between 6,227 matched centerline voxel pairs in panel E. (G) Aligning whole brain reconstruction vessel centerline voxels to the post perfusion reconstruction (Affine transformation, with linear scaling factors in three directions 1.0490, 1.0534, and 1.0510, respectively). (H) Histogram of residual displacement between 10566 matched centerline voxels in panel G.

3.7.2 Brain deformation correction

We also corrected for the overall brain deformation due to the sample preparation procedures. This global deformation is quantified by comparing the vascular images at different stages of the sample preparation with the *in vivo* images. In *Brain2*, part of the vasculature in the somatosensory cortex was imaged *in vivo* and right after perfusion before the brain was extracted. The vessel centerlines in post-perfusion images were aligned to the vessel centerline voxels in the *in vivo* images by similarity transformation to quantify the scale changes during perfusion. After whole-brain vasculature imaging and reconstruction, the final vessel centerlines were registered to the post-perfusion vessel skeleton by Affine transformation. The sample deformation in three dimensions was quantified by QR decomposition of the Affine transformation matrix. A very

uniform sample shrinkage was found in three orthogonal directions and therefore applied their geometric average (1.051) to correct the length scales (Figure 3.12). To further quantify the stability of the shrinkage, the same sample preparation procedures were applied to the brains used for vascular radius calibration experiments. For ten $180\ \mu\text{m} \times 180\ \mu\text{m} \times 400\ \mu\text{m}$ volumes in one brain, a linear scaling factor of 1.048 ± 0.007 was found. The extra sample deformation due to the delipidation process applied to *Brain 3* was quantified by registering its whole brain image stack to the whole brain image of *Brain 1* at $16\ \mu\text{m}$ isotropic resolution using similarity transformation in MATLAB. The resulting linear scaling factor 1.0926 was applied to correct the length scales.

3.7.3 Reconstruction quality quantification

To quantify the reconstruction quality of the microvascular network, six 240-cubes were randomly selected from cortical regions, thalamus, superior colliculus, and pons to reconstruct the microvascular network and visualized using itk-snap software [134] along with the image at 1-micrometer resolution. To highlight the vessel segments with unconnected endpoints inside the volume, unconnected endpoint voxels that are at least 1 voxel away from the cube edges were reconstructed as spheres and visualized in a separate color (green). Reconstruction errors, including unconnected segments, potential false positive connections due to the proximity of microvessels, sample preparation, and image processing, were detected by visually tracing the reconstruction mask overlaid on the image and inspecting the three-dimensional visualization. Among 2133 inspected segments, 1 false positive connection and 2 unconnected segments were found, i.e., 0.999 of the vessels are free of errors in connectivity (Figure 3.13).

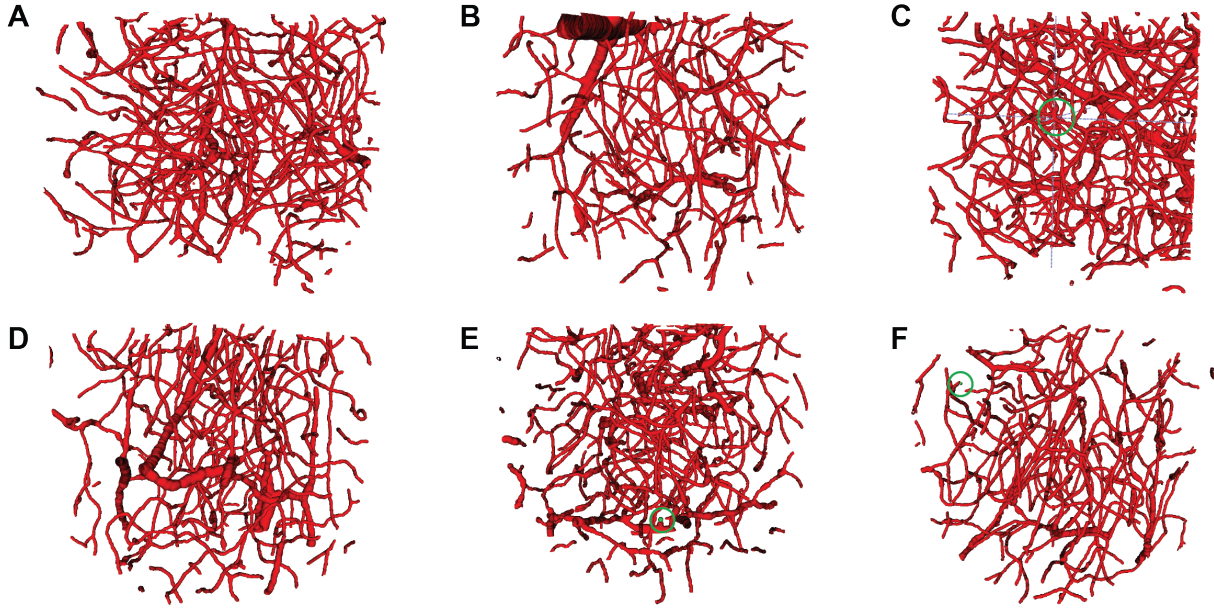


Figure 3.13. Visual Inspection of Reconstructed Vascular Network. The microvascular network is reconstructed as interconnected red curved cylinders in a $240 \times 240 \times 240 \mu m^3$ volume. (A) The 240-cube is randomly sampled from the primary somatosensory cortex and has 424 vessel segments and 208 nodes. No connectivity error is found. (B) Reconstruction in ventral auditory cortex with 306 branches, 148 nodes. No connectivity error is found. (C) Reconstruction in thalamus ventral nucleus with 421 branches, 199 nodes. One potential false positive connection between two capillaries is circled and localized by the blue cross-hair. (D) Reconstruction in the pontine reticular nucleus with 279 branches, 199 nodes. No connectivity error is found. (E) Reconstruction in superior colliculus with 381 branches, 186 nodes. One false positive branch with an unconnected endpoint (green) due to perfusion artifact was circled. (F) Reconstruction in the secondary motor cortex and anterior cingulate cortex, with 322 branches, 160 nodes. One branch with an unconnected endpoint (green) is circled.

3.8 Discussion

We chose to acquire data with serial section two-photon microscopy to maintain a stable signal-to-noise ratio and near-constant optical resolution with depth (Figure 2.3). The earliest techniques used counting of individual sections [8, 19], which led to surprisingly close estimates of the length density relative to more recent data (Table 3.1), but was limited to sampling selected regions rather than complete three-dimensional reconstructions. Tissue-clearing techniques and light sheet microscopy have been combined to generate whole mouse brain vasculature images with relatively short data acquisition times [22, 59, 77, 117, 135]. Yet light sheet microscopy

has reduced resolution when the wavefront of the excitation beam passes through expanses of imperfectly cleared tissue. While this arrangement accelerates image acquisition, it can lead to lower signal-to-noise ratios and potential overestimates of vessel diameter [59, 117] (Table 3.1). Lastly, the section-by-section micro-optical sectioning tomography-technique [69] has similar lateral but better axial resolution than two-photon imaging. However, this technique uses negative labeling so the segmentation may be complicated [132, 133]. Beyond segmentation issues, we calculated capillary radii based on optical deconvolution methods (Figure 3.7, Figure 3.6) and comparisons of identified live and labeled vessels (Figure 3.11).

Whole brain vascular reconstructions give insights into microvessels and their role as local supply chains (Table 3.1). Yet all efforts to date failed to preserve the complete connectivity back to the systemic circulation. This results from both the collapse and close opposition of surface arteries and veins [6] and the embedding of venous sinuses into the foramina [13, 44]. Moving forward will require an in situ analysis of the vasculature, a challenge that may be met with all optical histology [120] and/or x-ray tomographic techniques [25, 99, 128].

3.9 Acknowledgment

Chapter 2 to 4, in part, is a reprint of the material as it appears in X. Ji, T. Ferreira, B. Friedman, Rui Liu, H. Liechty, E. Bas, J. Chandrashekar, D. Kleinfeld, Brain microvasculature has a common topology with local differences in geometry that match metabolic load, *Neuron*, Volume 109, Issue 7, 2021, Pages 1168-1187.e13. The dissertation author was the primary investigator and first author of this paper.

Table 3.1. Comparison with related works. For Kirst et al 2020[59]: (1) Brain volume = $(3.2 \times 10^6 \text{ nodes per hemisphere} \times 2) \div (\text{average node density } 6400 / \text{mm}^3) = 1000 \text{ mm}^3$; (2) Total vessel length per brain = $(144 \pm 2) \text{ m per hemisphere} \times 2 = (288 \pm 4) \text{ m}$; (3) Average length density = $288 \text{ m} \div 1000 \text{ mm}^3 = 0.288 \text{ m/mm}^3$; (4) Average vessel radius is estimated from their Figure 4G, S3H and S6C; (5) Regional vessel length densities are from their Figure 4B or by averaging over the included sub-regions. For Todorov et al 2020 [117]: (1) Average vessel radius is estimated from their Figure 5C; (2) Regional length densities were computed from Supplemental data for C57BL/6 mice. For Xiong et al 2017 [133], regional length densities are estimated from their Figure 9E. For Boero et al 1999 [8] and Craigie 1920 [19], estimated regional length densities are averages of the included sub-regions.

		This study		Kirst et al 2020	Todorov et al 2020	Xiong et al 2017	Wu et al 2014	Blinder, Tsai et al 2013	Tsai et al 2009	Boero et al 1999	Craigie 1920
Data acquisition	Imaging	Serial two-photon microscopy		Lightsheet on hemisphere	Lightsheet on whole brain	MOST	MOST	Serial two-photon	Serial two-photon	Sections	Sections
	Voxel size (μm³)	0.30 x 0.30 x 1.0		1.6 x 1.6 x 1.6	1.6 x 1.6 x 3.0	0.35 x 0.35 x 1.0	0.35 x 0.40 x 1.0	1.0 x 1.0 x 1.0	1.0 x 1.0 x 1.0	-	-
	Labeling	Lumen perfusion (FITC)		Acta 2, CD31, Podocalyxin	Evans blue, wheat germ agglutinin	Modified Nissl staining	Modified Nissl staining	Lumen perfusion (FITC)	Lumen perfusion (FITC)	Nissl staining, silver emulsion	perfusion (carmin)
	Clearing	Index matching		iDISCO	3DISCO	-	-	-	Index matching	-	-
	Sample size	Whole brain		Hemisphere	Whole brain	Whole brain	Whole brain	Vibrissa cortex	Vibrissa cortex	-	-
	In vivo / in vitro comparison	Yes		-	-	-	Yes	-	Yes	-	-
Reconstruction	Segmentation resolution (μm)	1.0		1.6	3.0	0.35	0.35	1.0	1.0	-	-
	(μm)	0.25		1.6	3	0.35	0.35	1.0	1.0	-	-
	Reconstruction scale	Whole brain		Hemisphere	Whole brain	Selected regions	Selected regions	Vibrissa cortex	Vibrissa cortex	-	-
	Spatial graph representation	Whole brain		Hemisphere	-	-	-	Vibrissa cortex	-	-	-
Whole brain statistics	Graph refinement	Classifier-based		End-point tracing	-	-	-	Classifier-based	-	-	-
	Number of nodes (N)	4132583		3200000	-	-	-	-	-	-	-
	Number of branches (B)	6320303		4400000	-	-	-	-	-	-	-
	Average (B/N)	1.53		1.38	-	-	-	-	-	-	-
	Number of branches in the largest component	6238701		-	-	-	-	-	-	-	-
	Brain volume (mm³)	443		1000*	424	-	402	-	-	-	-
	Total length per brain (m)	384		288**	-	-	-	-	-	-	-
	(m/mm³)	0.867		0.288*	0.546	-	-	-	-	-	-
	Average vessel radius (μm)	2.4		4*	8*	-	-	-	-	-	-
	Regional vascular length density, Γ _L (m/mm³)		Vessel	Capillary	Vessel	Vessel	Capillary	Vessel	Vessel	Vessel	Capillary
Somatosensory cortex		1.01 ± 0.02	0.98 ± 0.02	0.41*	0.91 ± 0.09	-	-	-	-	1.07*	-
Vibrissa cortex		1.00 ± 0.03	0.97 ± 0.03	0.42*	0.63 ± 0.09	0.98*	0.88	-	0.88 ± 0.17	-	-
Auditory cortex		0.95 ± 0.01	0.92 ± 0.01	0.33*	0.77 ± 0.14	-	-	-	-	0.86*	-
Mortor cortex		0.93 ± 0.03	0.91 ± 0.03	0.37*	0.74 ± 0.12	-	-	-	-	0.87*	-
Visual cortex		0.85 ± 0.02	0.82 ± 0.02	0.33*	0.73 ± 0.11	-	-	-	-	0.78*	-
Hippocampus		0.71 ± 0.03	0.68 ± 0.02	0.30*	-	0.69*	-	-	-	-	-
Ammon's horn		0.68 ± 0.02	0.65 ± 0.02	0.32*	0.49 ± 0.02	-	-	-	-	-	-
CA1		0.66 ± 0.03	0.63 ± 0.02	0.33 ± 0.01	-	-	-	-	-	0.58*	-
CA3		0.71 ± 0.03	0.68 ± 0.03	0.33 ± 0.01	-	-	-	-	-	0.56*	-
Caudoputamen		0.85 ± 0.03	0.82 ± 0.03	0.41 ± 0.01	0.58 ± 0.06	-	-	-	-	0.49 ± 0.10	-
Thalamus		1.04 ± 0.08	1.00 ± 0.08	0.49*	-	0.95*	-	-	-	-	-
Hypothalamus		0.77 ± 0.03	0.74 ± 0.03	0.38*	0.49 ± 0.14	0.66*	-	-	-	-	-
Brainstem		0.97 ± 0.04	0.93 ± 0.04	0.46*	0.47 ± 0.05	-	-	-	-	-	-
Midbrain		0.97 ± 0.06	0.93 ± 0.05	0.43*	0.60 ± 0.04	-	-	-	-	-	-
Inferior colliculus		1.46 ± 0.04	1.41 ± 0.04	0.71 ± 0.01	0.81 ± 0.08	-	-	-	-	-	-
Superior colliculus		0.97 ± 0.06	0.92 ± 0.06	0.42*	-	0.88*	-	-	-	0.54*	-
Pons		0.97 ± 0.04	0.93 ± 0.03	0.54*	0.31 ± 0.06	-	-	-	-	-	-
Medulla		1.03 ± 0.02	0.98 ± 0.02	0.48*	0.36 ± 0.09	-	-	-	-	-	-
Medial vestibular nucleus		1.48 ± 0.08	1.41 ± 0.07	0.77 ± 0.02	-	-	-	-	-	-	1.36 ± 0.04
Dorsal cochlear nucleus		1.46 ± 0.08	1.39 ± 0.07	0.68 ± 0.03	-	-	-	-	-	-	1.47 ± 0.04
Superior olive		1.42 ± 0.04	1.37 ± 0.04	0.76 *	-	-	-	-	-	-	1.12 ± 0.04
Lateral vestibular nucleus		1.02 ± 0.19	0.98 ± 0.19	0.58 ± 0.02	-	-	-	-	-	-	0.93 ± 0.03
Cerebellum		1.10 ± 0.05	1.06 ± 0.04	0.63*	0.47 ± 0.05	-	-	-	-	1.20*	1.11*
Dentate nucleus		1.49 ± 0.12	1.45 ± 0.11	0.78 ± 0.08	-	-	-	-	-	-	1.27 ± 0.03

* Estimated from figures or other numbers reported in the referenced manuscript

**Estimated as twice the value per hemisphere

Chapter 4

Analyzing Mouse Brain Microvascular Network Connectome

4.1 Global structure of brain vascular network

The output of our pipeline is a vascular connectome with vessel centerline positioned at 1 μm resolution and the radii of vessels estimated at 0.25 μm resolution (Figure 4.1A). An abstracted graph of edges and nodes, with the edges labeled by the length of the centerline and the radius found as the median of the radii for all centerline voxels (Figure 4.1B), can be derived from the connectome. On average, $(278 \pm 12) \times 10^6$ (mean $\pm$ SE) centerline voxels form an undirected graph with $(6.32 \pm 0.27) \times 10^6$ edges (E) and $(4.13 \pm 0.17) \times 10^6$ nodes (N). We obtained 384 ± 16 m of vessel segments filling 443 ± 9 mm³ of brain tissue after correcting for tissue shrinkage during the sample preparation (Figure 3.12). The largest graph connected component contains $98.7 \pm 1.2\%$ of the edges. Thus, virtually all of the reconstructed vessel segments form an interconnected network.

Detailed statistics for the vascular connectomes are summarized in Table 3.1 and Table 4.1. It's worth emphasizing that this result is from completely automatic processing of the entire imaged volume, and therefore contains regions with numerous segmentation errors, including the space outside the brain and the choroid plexus. However, most of the following analyses were based on local statistics inside the brain, independent of these outlier regions.

Microvessels dominated the probability density function (PDF) of branch radius (Fig-

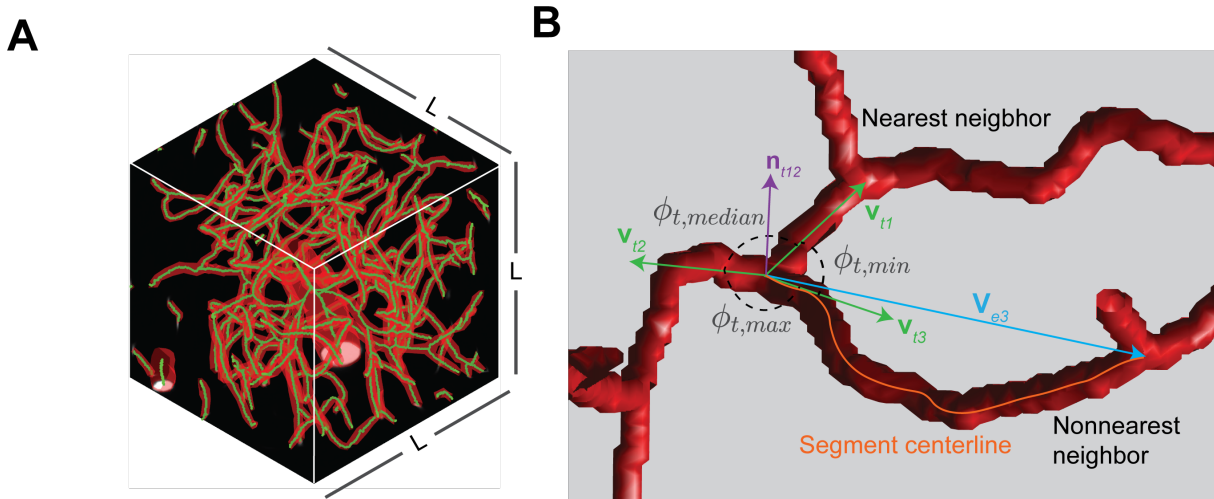


Figure 4.1. Visualization of local vascular network reconstruction. (A) Reconstructed vessel mask (red tubes) with centerlines (green curves) in one 240-cube. (B) Reconstructed rendering of a small segment of the vascular network highlighting angles at a triadic node.

Table 4.1. Whole-brain-wide reconstructed graphs statistics. SD is the standard deviation. CV is the coefficient of variation.

	Brain1	Brain2	Brain3	Average	SD	CV
Brain volume (mm ³)	443	434	452	443	9	0.02
Number of skeleton voxels	283710360	264432433	285710552	2.78E+08	1.18E+07	0.04
Total segment length (m)	395	365	392	384	16	0.04
Number of branches, B	6571475	6036661	6352772	6.32E+06	2.69E+05	0.04
Number of nodes, N	4284051	3948612	4165085	4.13E+06	1.70E+05	0.04
B/N	1.534	1.529	1.525	1.529	0.004	0.003
Fraction of branches in the largest graph component	0.995	0.973	0.993	0.987	0.012	0.01
Length density (m/mm ³)	0.892	0.842	0.867	0.867	0.025	0.03
Lateral resolution, ω_{xy} (μ m)	0.40 $\pm$ 0.02	0.48 $\pm$ 0.02	0.41 $\pm$ 0.02	-	-	-
Axial resolution, ω_z (μ m)	3.18 $\pm$ 0.17	3.82 $\pm$ 0.21	2.84 $\pm$ 0.09	-	-	-

ure 4.2A), as anticipated. The PDF follows a power law decrement for radii larger than 3.5 μ m (Figure 4.2A). The asymptotic power law has a scaling exponent of -2.81 ± 0.03 with $R^2 = 0.99$ for radii larger than 3.5 μ m (Figure 4.2A). This exponent is close to that of -3 given by Murray's Law to minimize the cost required to drive the flow of liquids through a network of tubes and concurrently maintain the transportation system [84].

We classified vessel segments as capillary versus non-capillary based on the threshold radius of 3.5 μ m for deviation from a power law; this will be further justified by additional analysis. Under this definition, capillaries comprise about 80% of the vascular volume with each

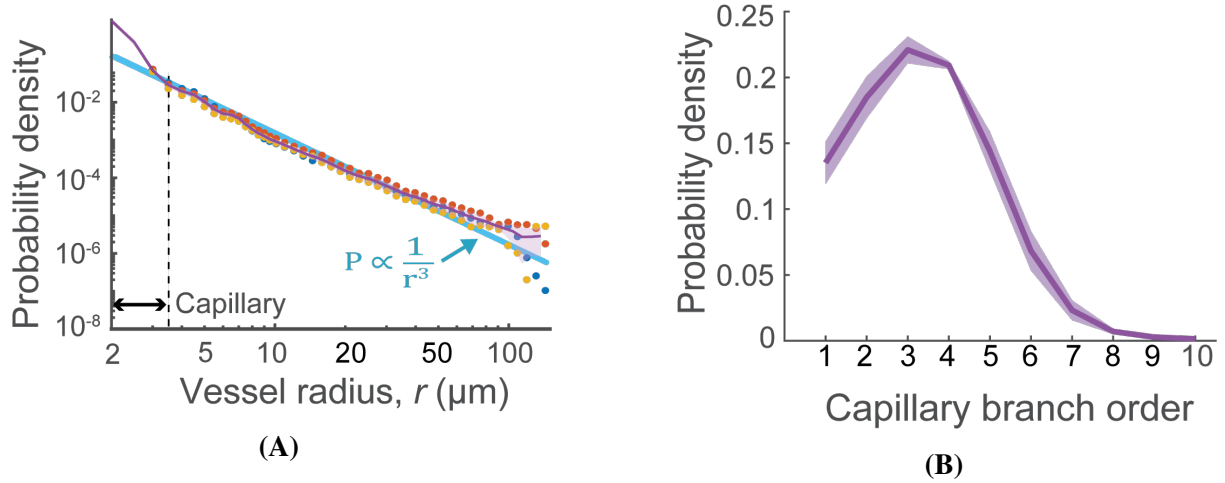


Figure 4.2. Vessel radius and capillary order distributions. (A) The PDF of the radius distribution for all vessels for all three brains (red, yellow, and blue dots); the mean (solid) and standard deviation (shadow) are in purple. The mean value of the radius is $2.39 \pm 0.08 \mu\text{m}$ (mean $\pm$ SD) and the median is $2.05 \pm 0.05 \mu\text{m}$. The power law (blue line) has a slope of -3. (B) The PDF of the capillary branch order from the nearest penetrating vessels as an average over three brains.

240-cube (Figure 4.3) and account for the vast majority, i.e., 95%, of the total vascular length.

Starting from the non-capillaries, i.e., penetrating arterioles or venules, we iteratively labeled edges according to the geodesic distance to the nearest non-capillary segment. The capillary edges that are directly connected to non-capillaries were labeled as order 1, the unlabeled edges that are directly connected to order 1 edges were labeled as order 2, and so forth. Most of the capillaries are of order 3 - 4, with an average branch order of 3.4 ± 0.2 (mean $\pm$ SD) (Figure 4.2B), while few are more than 7 branches from the major vessels, as shown previously [59]. Assuming arteries and veins are positioned interleavingly, then on the whole brain scale, the small branch order suggested that the clusters of major vessels are distributed relatively uniformly in the brain, with the shortest path from arteries to veins containing on average 7 capillary segments.

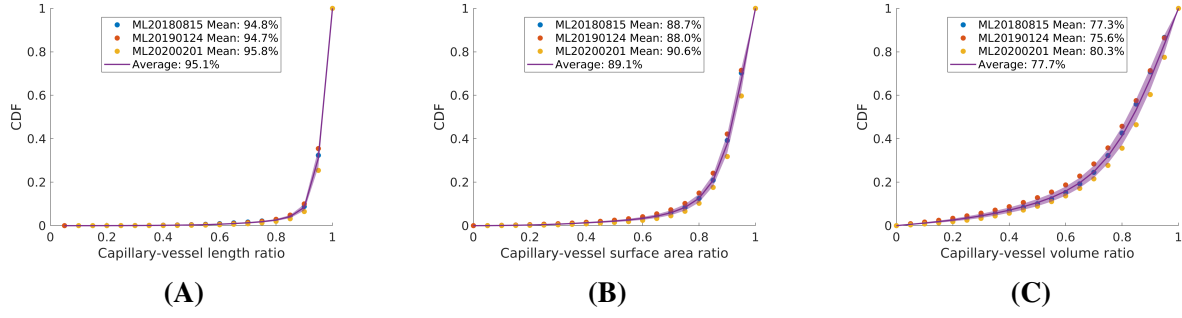


Figure 4.3. Cumulative density functions of capillary-to-all-vessel ratio in all the 240-cubes within the brains. (A) Ratio between the total capillary length and the total vessel length within the 240-cubes. (B) The ratio between the total capillary surface area and the total vessel surface area within the 240-cubes. (C) The ratio between the total capillary volume and the total vessel volume within the 240-cubes.

4.2 Systematic Quantification of Brain Microvascular Network Structure

4.2.1 Brain microvasculatures have a common network topology

The primary topological feature is the number of edges that join at each node, defined as the degree of the node. Across all 240-cubes, we found that edges were dominantly jointed as triads, with 96.8% to 98.7% (middle quartiles) of the nodes being of degree 3 and an average degree 3.02 ± 0.02 across all brains. As a result, the branch-to-node ratio of 1.529 ± 0.004 (mean $\pm$ SD) is very close to the theoretical value of $3/2$ for an infinite mesh graph of degree 3. Notice that our definition of node differs from the conventional definition in graph theory that includes unconnected edge endpoints (free-ends). Under our definition, the theoretical ratio of network with N nodes of averaged degree $\bar{D}$, E edges and F unconnected endpoints in a single connected component is $\frac{\bar{D}}{2} + \frac{F}{2N}$. With these statistics, we concluded that virtually all edges are in a single connected component and virtually all edges connect exclusively as triads. Thus vessels form a highly redundant network with roughly $N/2$ independent loops, where the number of independent loops is the maximum number of edges that can be deleted before the network breaks into two connected components [86].

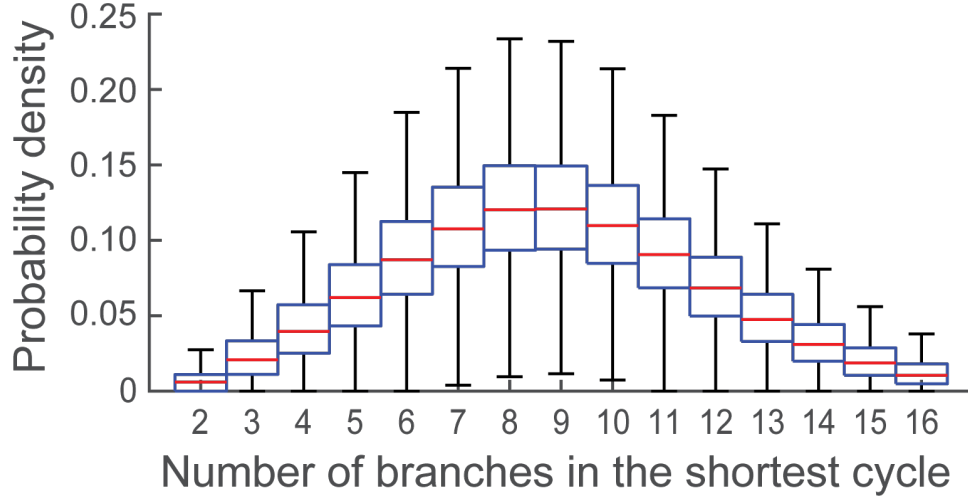


Figure 4.4. The probability density function (PDF) of the number of branches in the shortest cycle of vessels. Each whisker shows the distribution of the probability density in the bin for all the 240-cubes within the brains, outliers are not shown, same as below.

Network topology can be further quantified by the number of edges in the shortest path that one needs to traverse to return to the same node. Relatively consistent distributions were found across the whole brain, with 8.97 ± 0.53 (mean $\pm$ SE) edges in the shortest loop (Figure 4.4). Only about 0.011 (median) of the loops consist of only two edges, i.e., edges in parallel, which suggests the low frequency of matured brain vessels originates from intussusceptive angiogenesis.

The interplay between Euclidean and geodesic distance, i.e., the minimum number of edges, between nodes indicates the compactness of the connectivity. We computed the pairwise Euclidean distance between pairs of nodes and counted the number of edges between the pairs. We find that 85% to 91% of the nodes were directly connected to their nearest node, while the other edges form non-nearest neighbor connections.

4.2.2 Topological basis of common network robustness

Spatial dimension and network topology are primary factors that determine bond percolation threshold for a regular space-filling network[127]. Consequently, similar microvascular network topology implies a shared bond percolation transition threshold across different brain

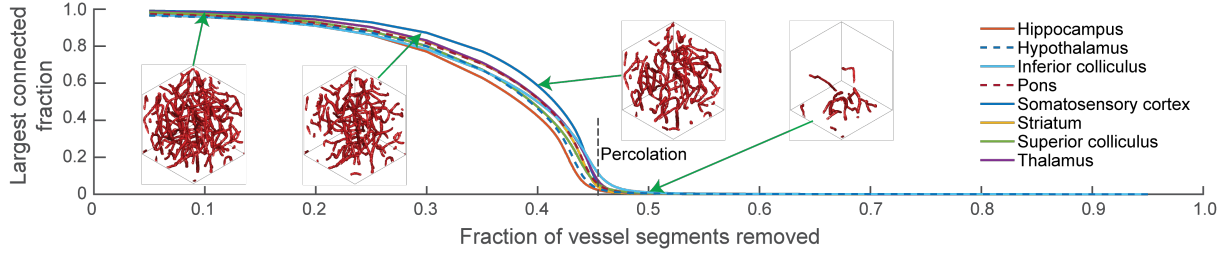


Figure 4.5. Bond percolation transition of brain microvascular network. Catastrophic disassembly of regional vascular networks after 44% of the vessel segments were randomly removed. Inserts were examples of the largest graph connected component visualized in a 240-cube. The standard deviation of the largest connected fraction is less than 0.02 for cases with less than 0.4 or greater than 0.5 of the vessel segments removed. The standard deviation increases to 0.05 near the threshold for disassembling the network. Brain regions were assigned using the Common Coordinate Framework [129].

regions. To computationally verify this prediction, we constructed the abstracted undirected vessel graph for each brain region in each hemisphere, randomly removed different fractions (p) of vessel segments (edges), and computed the fraction of remaining nodes in the largest graph connected component $s(p)$. The process was repeated 500 times for each removal fraction and each region. In all the brain regions, the largest component rapidly broke into small graphs when 43.3 % to 44.7% of the segments were removed (Figure 4.5), where the transition threshold was found as the fraction that maximized $|ds/dp|$. Therefore, the bond percolation threshold was about 0.56. This is very close to the known bond percolation thresholds of 54.2% to 55.6% for triadic lattices [119]. Thus, as a result solely of topology, the loss or stall of less than half of all capillaries will lead to a catastrophic failure of the vascular system.

4.2.3 Common branching geometry of vascular network

What is the branching geometry of the triads? If we join three randomly oriented straight lines in three dimensions, the distribution of angles between lines would fall into a unit sphere. To determine if this holds for the angular distribution of the vasculature, we computed the end-to-end vector $\mathbf{v}_e$, endpoint tangent vectors $\mathbf{v}_t$, and the length l of each vessel segment (Figure 4.1B). First, the ratio between the vessel length and the end-to-end distance $l_e = |\mathbf{v}_e|$, i.e., the tortuosity

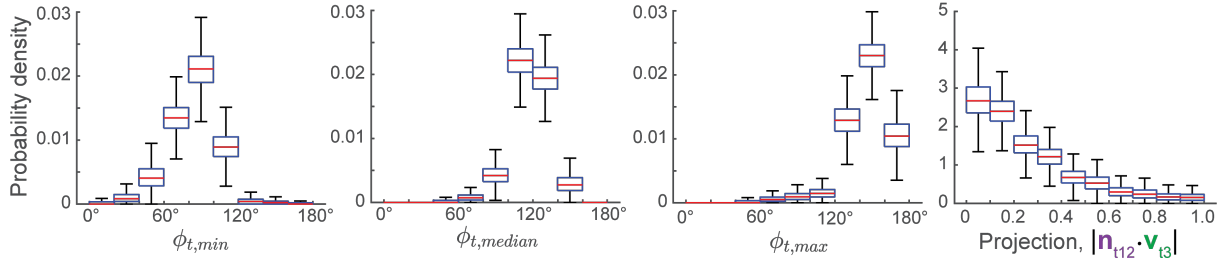


Figure 4.6. PDFs of branch angles distributions The PDFs for the smallest, median, and largest branch tangent angle of vessels joined at a node, along with the cosine of the elevation angle between the third tangent vector and the plane spanned by the first two tangent vectors. The average value of $\phi_{t,min}$, $\phi_{t,median}$, and $\phi_{t,max}$ are $84 \pm 4^\circ$, $118 \pm 2^\circ$, and $145 \pm 3^\circ$ (Mean $\pm$ SE), respectively.

of the branch, is 1.27 ± 0.05 (mean $\pm$ SE) as an average over all 240-cubes (**Figure 3C**). Thus, vessels in the brain are fairly straight. Using the branch tangent vectors near the node, the average minimum, median and maximum angle between edges, denoted as $\theta_{t,min}$, $\theta_{t,median}$, and $\theta_{t,max}$, were found to be $84 \pm 4^\circ$, $118 \pm 2^\circ$, and $145 \pm 3^\circ$, respectively (Figure 4.6). As two linearly independent vectors defined a plane with the normal vector $\mathbf{n}_{12}$ (Figure 4.1B), we also computed the absolute value of its projection onto the third vector. The average projection was 0.255 ± 0.037 and corresponds to a small average elevation angle of 14.8° . This implies that edges tend to stay in a plane near the branch point (Figure 4.1B).

4.3 Brain microvascular network anisotropy

Before high-resolution 3D imaging and reconstruction became available, the vascular density was usually estimated from the area ([9]) or the skeleton length ([130]) of vessels on thin slices. Except for the underestimation from overlapping vessels, uniform random orientation of the vessels in space (isotropic) is a crucial assumption for converting 2D projections to 3D density estimation. However, brain is an organism with complex structure and developmental dynamics, and the white matter regions contain nerve tracts organized in a highly anisotropic manner. As blood vessels and axons share common developmental cues ([14]), it's not obvious that brain vessels is in general isotropic. In fact, it has been well-known and recognized that the major

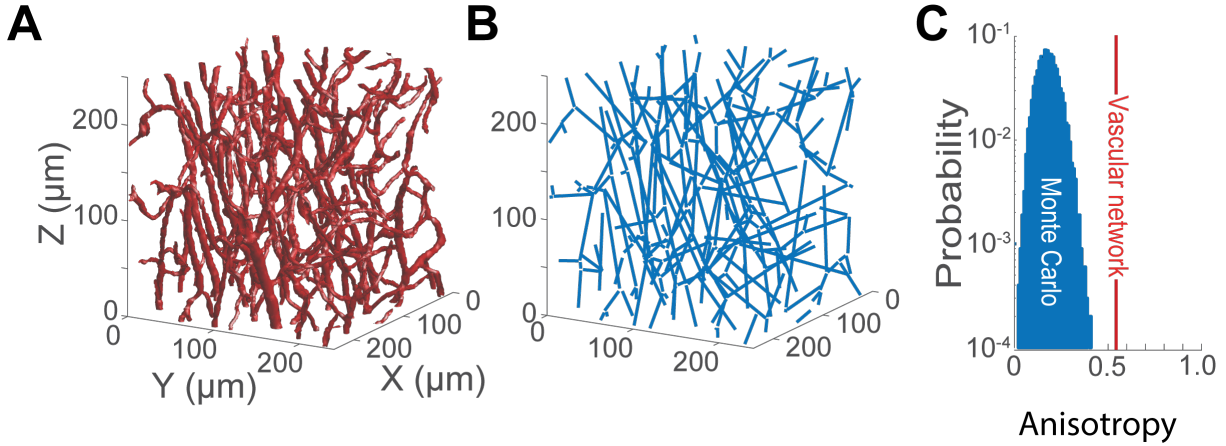


Figure 4.7. Highly anisotropic brain microvascular network (A) The reconstructed vascular network in a 240-cube. (B) Abstracted vessel segments drawn as endpoint-to-endpoint straight lines. (C) Volume-weighted local vascular network fractional anisotropy computed from the lines in panel B (red vertical line) and the distribution of anisotropy computed from the same number of randomly orientated vessels with the same volume weights (blue histogram; 10000 trials).

vessels in different regions have specific structures and their orientation is far from isotropic. For example, in the cortical region, pial vessels cover the cortical surfaces, dominating local volume-weighted network orientation. Underneath, the diving arteries and ascending veins are approximately perpendicular to the brain surface. Then, what about capillaries? Until recently, it has been believed that the capillary network in the brain exhibits little orientation preference and is, therefore, statistically isotropic ([32, 113]), which is also an underlying assumption for modeling the blood oxygen level dependent (BOLD) signal ([30, 90]). However, there is little experimental evidence that directly quantifies the anisotropy of the capillary network, while Hernandez et al ([45]) show that vessels in the white matter are preferentially orientated along the fiber tracts, and the cerebral blood flow (CBF) measured in human subjects varies up to 130% as the relative orientation of the main magnetic field changes.

4.3.1 Computing local network anisotropy

We systematically quantified the local capillary network statistical anisotropy throughout the brain by computing the fractional anisotropy (FA) derived from the eigenvalue of the

correlation matrix of the volume-weighted vessel segment orientation vector.

The end-to-end vectors of the vessel segments inside the 240-cubes (Figure 4.7A) were used to quantify the network anisotropy. The end-to-end vector represents the net orientation of the vessel segment, as it equals the integral of the vessel tangent vector along the segment (Figure 4.7B). The volume of a 240-cube is about an order of magnitude larger than the inter-vessel distance, while relatively small compared to the brain structures.

For vessel segment i , the volume-weighted orientation vector was $V_i \mathbf{v}_e^{(i)}$. As the order of two segment endpoints was assigned randomly, $-V_i \mathbf{v}_e^{(i)}$ was also the volume-weighted orientation vector of segment i . To remove the randomness in endpoint assignment, the volume-weighted orientation matrix of the N vessels was a $2N$ -by-3 matrix with each column sum to 0:

$$M = \begin{bmatrix} V_1 (\mathbf{v}_e^{(1)})^T \\ \dots \\ V_N (\mathbf{v}_e^{(N)})^T \\ -V_1 (\mathbf{v}_e^{(1)})^T \\ \dots \\ -V_N (\mathbf{v}_e^{(N)})^T \end{bmatrix} \quad (4.1)$$

The 3-by-3 correlation matrix C was computed as $M^T M / (2N - 1)$, from which the three eigenvalue λ_1 , λ_2 and λ_3 were obtained. The preferred orientation of the local vascular network was given by the eigenvector corresponded to the largest eigenvalue, while fractional anisotropy (FA) was used to quantify the absolute anisotropy level of the network:

$$FA = \sqrt{\frac{1}{2} \frac{\sqrt{(\lambda_1 - \lambda_2)^2 + (\lambda_2 - \lambda_3)^2 + (\lambda_3 - \lambda_1)^2}}{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}} \quad (4.2)$$

For isotropic network, as $N \rightarrow \infty$, $\lambda_1 = \lambda_2 = \lambda_3 = 1/3$, and therefore $FA = 0$, while if all the vessel segments were along one direction, $FA = 1$. For finite N , the statistical significance of

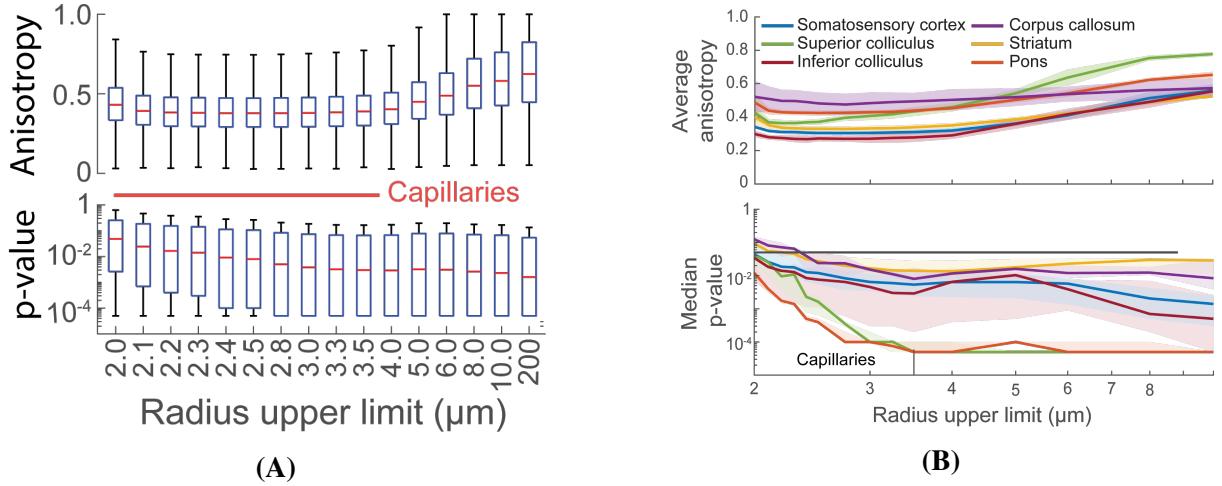


Figure 4.8. Whole brain vascular network anisotropy versus upper radius cutoff. (A) The anisotropy distribution across 240-cubes in two brains, along with statistically significant (p-value), as a function of the maximum vessel radius included. The median p-value is $p < 0.01$ down to radii of $2.4 \mu\text{m}$. All p-values smaller than 10^{-4} are set to 5×10^{-5} for visualization purposes, same as panel Figure 4.8B, Figure 4.10, and Figure 4.9. (B) The radius-dependence of the average anisotropy, including standard error bands, and associated median p-values, including 25% to 75% bounds over 4 hemispheres.

network anisotropy was quantified by Monte Carlo sampling (Figure 4.7C). The same volume weights were assigned to N unit orientation vectors randomly sampled from the surface of a unit sphere to compute FA. The sampling was repeated 10000 times, and the resulting null hypothesis distribution was used to compute the z-score FA_z and p-value FA_p of the local vascular network. For visualizing p-value in log scale, $FA_p < 10^{-4}$ was set to be 5×10^{-5} .

4.3.2 Region-specific microvascular anisotropy

On the whole brain scale, as an average across all of the 240-cubes, the anisotropy was lowest, yet statistically significant and relatively constant at ~ 0.4 when vessels with radius at or below $3.5 \mu\text{m}$ were considered (Figure 4.8A); this corresponds to our cut-off for capillaries (Figure 4.2A).

The regional capillary anisotropy was visualized as an overlay in horizontal and sagittal projections, color-coded with the p-value to indicate statistical significance (Figure 4.9, Online Video). The boundaries of brain regions were determined by registering brains to the Common

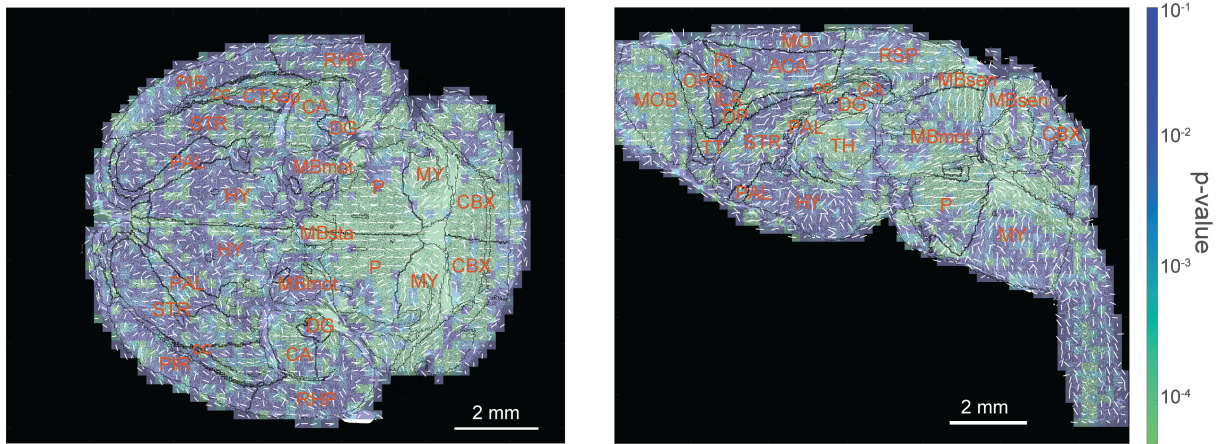


Figure 4.9. Visualization of whole brain microvascular network anisotropy. Local capillary (radius $\leq 3.5 \mu\text{m}$) anisotropy statistical significance (p-value) and preferred orientation (white lines) overlaid on a 240- μm thick maximum-projection of reconstructed vessels. Black curves and red labels indicate regional contours and name abbreviations according to Allen Atlas. Full names for the abbreviations are summarized in Table 4.3.

Coordinate Atlas [129]. Particularly striking anisotropy was observed within the superior colliculus along the dorsal–ventral axis, and within the pons along the caudal–rostral axis (Figure 4.8B). The corpus callosum also had high anisotropy, albeit with low statistical significance as a result of its relatively low density of vessels. Contrariwise, the inferior colliculus had low yet significant anisotropy. The anisotropy with neocortex varied with depth. Capillaries were found to preferentially lie normal to the pial surface within the superficial layers, yet were only weakly oriented within the deep layers of cortex (Figure 4.10). These results emphasized the importance of microvascular network structure in interpreting high-resolution fMRI signals across brain regions ([4]).

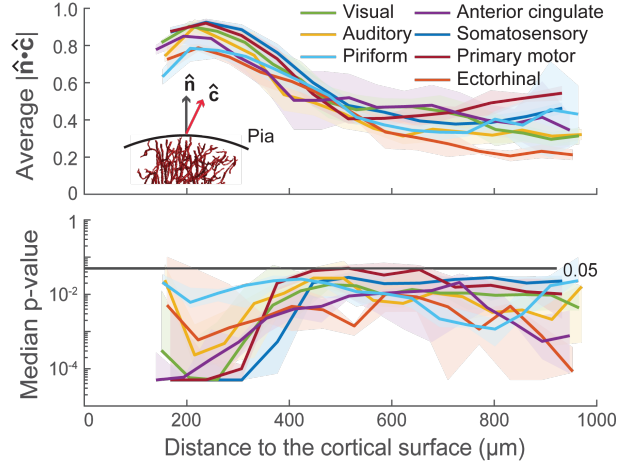


Figure 4.10. Cortical depth-dependent microvascular anisotropy Cortical depth-dependence of the average orientation of capillaries, including standard error bands, and associated median p-values within a region, including 25 % to 75% bounds. The plotted data is the mean orientation (c) projected onto the normal to the cortical surface (n) as an average over 4 hemispheres.

Given the highly organized neural structures revealed by diffusion tensor imaging [82] and the shared developmental mechanism of nerve and vessels [14], it is not surprising that a large fraction of brain vascular network is nearly anisotropic down to capillary level (Figure 4.8B). These orientation preferences have been described in cortex [15, 59] and human white matter [88], while recent studies have shown the profound impact of cortical vessel anisotropy on magnetic resonance imaging [4, 32, 45, 125]. Our systematic quantification of vessel anisotropy is consistent with past observations in cortex [15, 59], provides structural evidence for the orientation-dependent MRI signal in white matter [45, 125], and reveals strongly orientated vascular network in superior colliculus and pons (Figure 4.8B).

4.4 Descriptive statistics of regional capillary length

Different brain regions have different susceptibility to microstrokes and varying energetic loads. As the first step to elucidate the role of potential vascular geometry in explaining these differences, we examine the length density, denoted ρ_L , of capillaries. Qualitatively, selected brain slices indicate strong regional differences with a relatively high length density in neocortex,

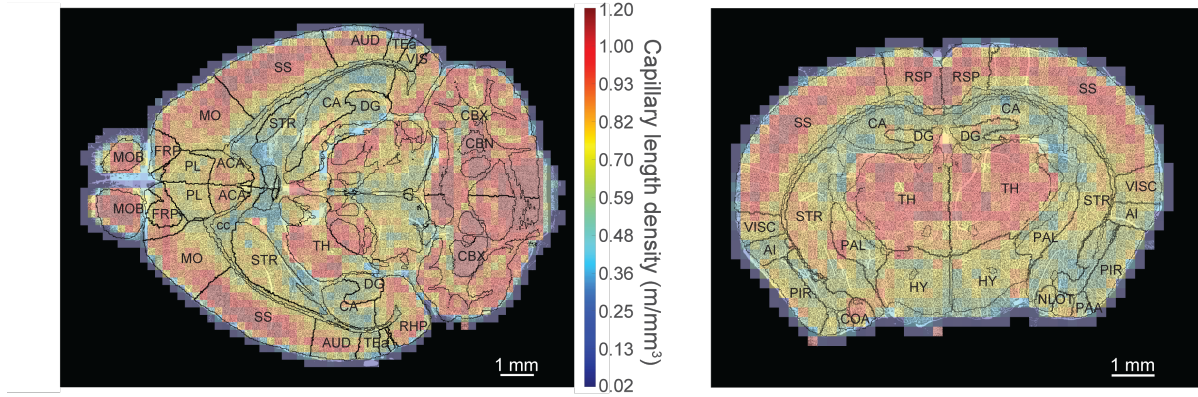


Figure 4.11. Visualization of whole brain capillary density. Local capillary length density overlaid on a 240- μm thick maximum-projections of reconstructed vessels. Full names for the abbreviations are summarized in Table 4.3.

thalamus, and cerebellum (Figure 4.11, Online Video). We next compute the PDF of ρ_L in each major brain region as an average across hemispheres (3 mice) (Figure 4.12A, Table 3.1 and Table 4.2). Three-fold differences in length density are observed, from $\rho_L = 0.49 \pm 0.12 \text{ m/mm}^3$ (mean $\pm$ SE) in corpus callosum, a region dominated by white matter, to $1.41 \pm 0.05 \text{ m/mm}^3$ in inferior colliculus, an auditory nucleus known for high neuronal activity [38].

The variation in capillary length density across the cortical mantle is modest. Neocortical regions, like somatosensory cortex, had higher average density than paleocortex regions, like piriform cortex, i.e., $\rho_L = 0.98 \pm 0.02 \text{ m/mm}^3$ (mean $\pm$ SE) versus $0.72 \pm 0.01 \text{ m/mm}^3$, respectively. Our values for neocortical regions are consistent with all but one past study (Table 3.1). Further, and consistent with past work [6, 59, 121, 132], the density within a multitude of neocortical areas varied weakly as a function of depth (Figure 4.12B). There is a peak in ρ_L at approximately 400 μm below the pia for auditory, somatosensory, and visual areas, albeit no discernible peak for anterior cingulate cortex. In contrast to neocortex, the density in piriform cortex was highest in the superficial layers and decreased to a minimum value at approximately 400 μm below the pia (Figure 4.12B).

The difference in length density across regions is related to the difference in the distribution of the length of different capillary edges. Regions with high values of ρ_L , such as

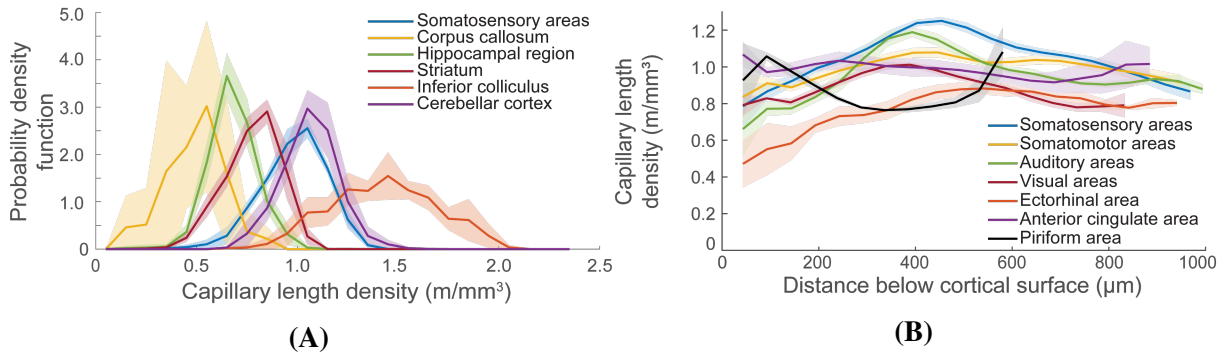


Figure 4.12. Region-specific capillary length density. (A) The PDF of capillary length density of 240-cubes in selected brain regions. Solid lines and transparent shadow show mean and standard deviation (SD) for six hemispheres, the same as panel D. (B) The depth dependence of the capillary length density within different regions of neocortex and piriform cortex. Solid lines and transparent shadows show mean and standard error (SE) for six hemispheres.

inferior colliculus and somatosensory cortex, have relatively more short edges, with median lengths of $45 \pm 1 \mu\text{m}$ (mean $\pm$ SE) and $46 \pm 1 \mu\text{m}$, respectively. The corpus callosum, with relatively low density (Figure 4.12A), has significantly longer median branch lengths, i.e., $67 \pm 3 \mu\text{m}$. Interestingly, edges shorter than $40 \mu\text{m}$ dominate across the brain, while edges that extend beyond $200 \mu\text{m}$ are rarely observed (Figure 4.13A, Figure 4.13B). In all regions, the segment length distributions are all positively skewed, and interestingly, the shorter half of the vessel segments only account for about 20% of the total length in the region, while longer segments up to hundreds of micrometers contribute to 80 % of the total length. All told, regions with higher capillary density have more short edges, and vice versa.

4.5 Scaling relations between vessel density and tissue-to-vessel distances

4.5.1 Quantifying tissue-to-vessel distances

An important metric to relate angioarchitecture to physiology is the distribution of distances from any point in the parenchyma to the lumen of the nearest vessel. We consider all vessels in our analysis, as substrates are available from penetrating vessels as well as capillaries

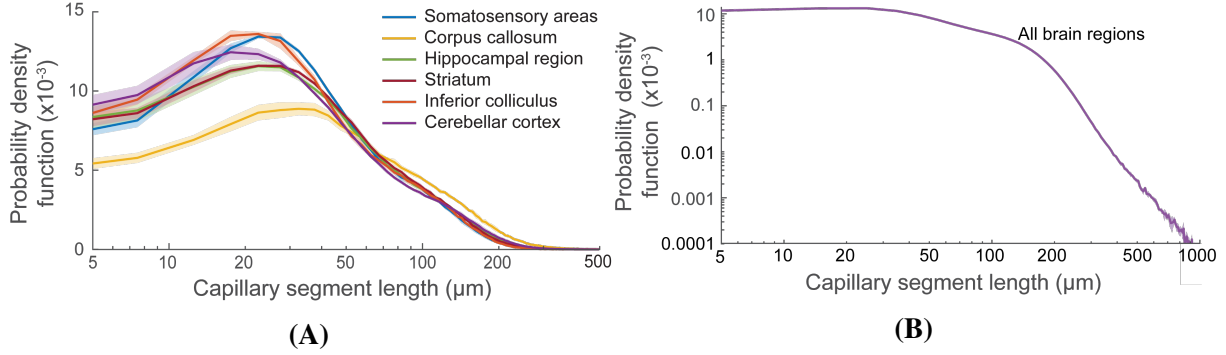


Figure 4.13. Capillary segments length. (A) The PDF of the length of individual capillary branches within selected brain regions. Regions with higher vessel density have more short vessel segments. (B) The PDF of the length of individual capillary branches in the entire brain. Solid line and transparent shadow show the mean and standard deviation for 3 brains.

[48]. We quantified the space-filling properties of the vascular network by computing the distance map, denoted $d(\mathbf{r})$, between tissue at location $\mathbf{r} = (x, y, z)$ and its nearest vessel wall (Figure 4.14). The mean distance between tissue voxels and their nearest vessel voxels denoted $\bar{d}$ as found by averaging $d(\mathbf{r})$ over each 240-cube, increases from $\bar{d} = 10.0 \pm 0.20 \mu\text{m}$ (mean $\pm$ SE) in inferior colliculus to $20.2 \pm 3.9 \mu\text{m}$ in corpus callosum (Figure 4.15A). Thus locations in a region with a high length density of capillaries lie relatively close to a vessel (cf Figure 4.12A versus Figure 4.15A), and vice versa.

The vasculature must supply energy substrates to cellular structures that occupy all of the space between vessels. Thus a physiologically important analysis is to determine the relation between the set of local, maximum distances to each neighboring vessel and the vessel length density across different brain regions. To address this goal, we searched for local maxima in the distance map, denoted $\bar{d}_{max}$, and computed the average value of the local maxima (Figure 4.15B). The PDF for $\bar{d}_{max}$ across different brain regions yields values that are about twice as large as for the PDF of $\bar{d}$ (Figure 4.15A, Figure 4.15B).

4.5.2 Tissue-to-vessel distances scale with vessel length density

We seek a quantitative relation between the distance map and the length density. As scaling laws should depend on the dimensionality but not details of the lattice, we consider first

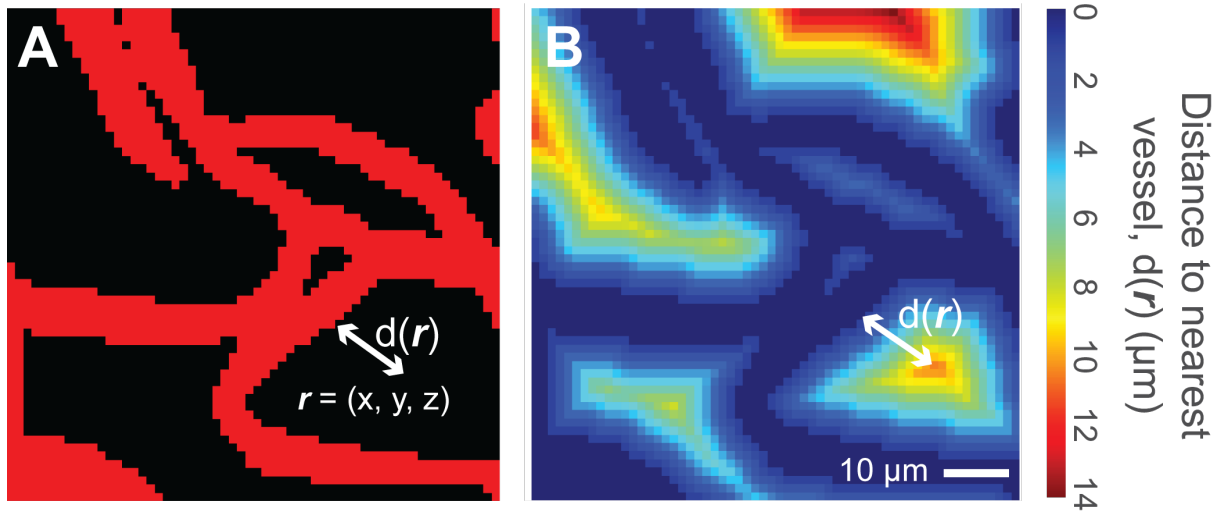


Figure 4.14. Visualization of whole brain capillary density. (A) 2D vessel mask (red). (B) The distance map, $d(\mathbf{r})$, with respect to vessel segmentation. This determines the distance between tissue at $\mathbf{r}$ and the nearest vessel wall.

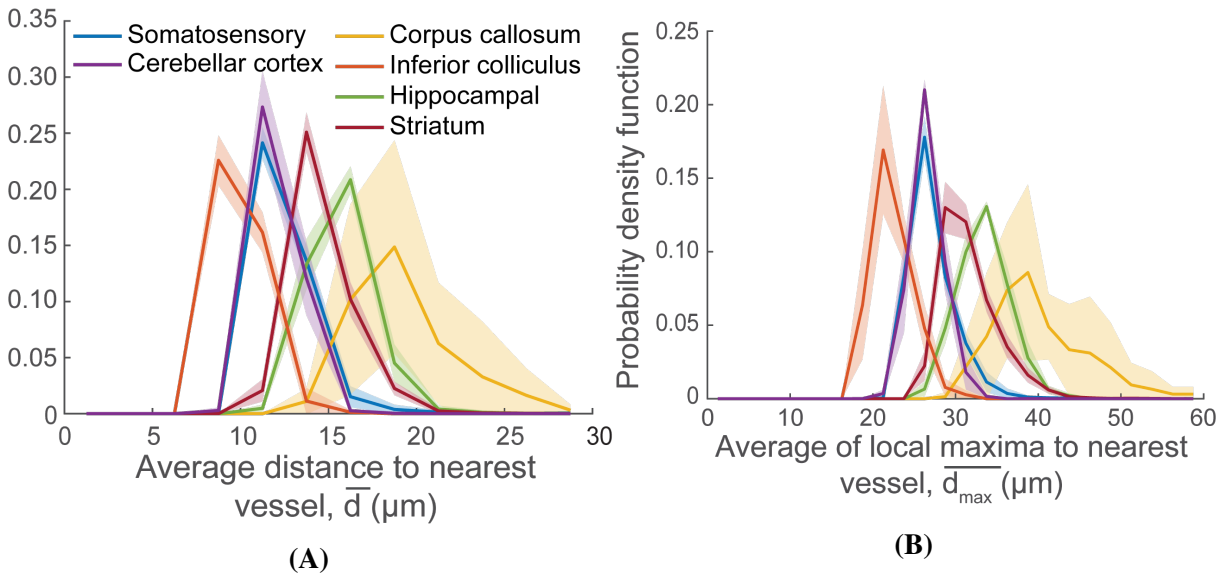


Figure 4.15. Average distances between tissue and the nearest vessel wall. (A) The PDF of the average distance between the tissue and the nearest vessel wall in selected brain regions. (B) The PDF of average tissue-to-vessel distance local maxima $\bar{d}_{max}$ in selected brain regions. Local maxima were found by moving a maximum filter with a window size of $50 \mu\text{m}$, which exceeds the fall-off in $\bar{d}$, and averaged over 240-cubes.

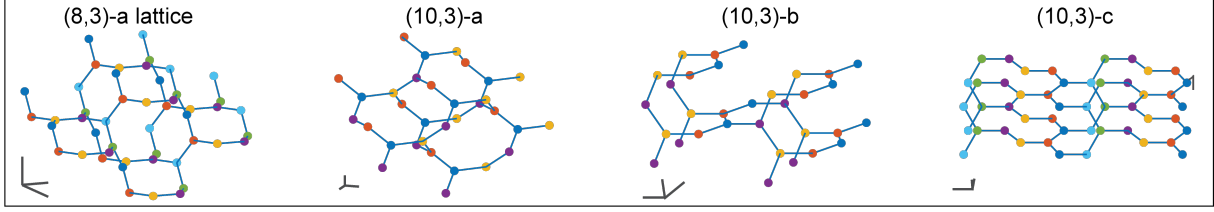


Figure 4.16. Degree-3 regular space-filling networks. Structures of 4 space-filling lattices with 3 nearest-neighbor connections, uniform edge length, and a common branch angle of 120° . The number of branches in the shortest loop for a $(k, 3)$ lattice is k . Points of the same color in each lattice are related by translation.

the exact result for a cubic lattice where each branch has the same length l that fills a chunk of tissue of length L . The volume of the tissue is L^3 , the total length of the vessels is $L_{tot} = 3L^3/l^2$, and the vessel length density ρ_L is therefore $3/l^2$. The average distance between any point in the space to the nearest edge could be calculated as:

$$\bar{d} = \frac{\int_0^{l/2} dx \int_0^{l/2-x} dy \int_0^{l/2-x} dz \sqrt{y^2 + z^2}}{\int_0^{l/2} dx \int_0^{l/2-x} dy \int_0^{l/2-x} dz} = \frac{\sqrt{2} + \sinh^{-1}(1)}{8} l, \quad (4.3)$$

and therefore

$$\bar{d} = \frac{\sqrt{3}(\sqrt{2} + \sinh^{-1}(1))}{8} \rho_L^{-1/2} = 0.497 \rho_L^{-1/2} \quad (4.4)$$

As for the maximum distance between space and its nearest network segment, its value is maximized at the center of each cube. The DT maximum is $d_{max} = \sqrt{2}l/2$, and its relation with vessel length density is:

$$d_{max} = \sqrt{\frac{3}{2}} \rho_L^{-1/2} = 1.225 \rho_L^{-1/2} \quad (4.5)$$

Note that for all these regular lattices, $d_{max} = \bar{d}_{max}$.

Though derived for cubic lattice, both scaling relations should hold for any 3D regular lattices [119] (Figure 4.16). Specific network geometry only affects the slope, which measures how well a network can reduce the distance between tissue and vessels, given the same length density.

Interestingly, we find that the irregular vascular network, where the irregularity originates

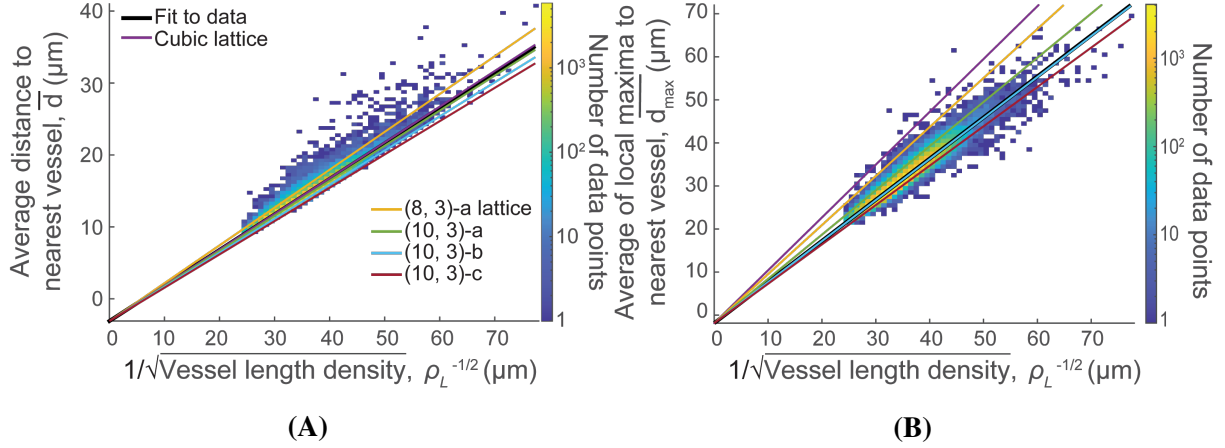


Figure 4.17. Geometric scaling relations of vascular network geometry. (A) Average vessel-tissue distance scales as vessel length density, i.e., $\bar{d} = m\rho_L^{-1/2} - 3.08$ with slope $m = 0.494$; $R^2 = 0.95$. The data encompasses 91,751 240-cubes where capillaries accounted for the majority of the vascular volume. The slopes for (8, 3)-a, cubic, (10, 3)-a, (10, 3)-b, (10, 3)-c lattices are $m = 0.526, 0.497, 0.487, 0.474$ and 0.463 , respectively. For regular degree-3 lattices, the slopes for the scaling relations are numerically computed from their three-dimensional reconstruction. (B) Scaling relation between the maximum vessel-to-tissue distance and the vessel length density. Regression for the vascular network was the same in panel Figure 4.17A, with $n = 0.955$ and $d_0 = 1.85 \mu\text{m}$; $R^2 = 0.94$. The slopes for cubic, (8, 3)-a, (10, 3)-b, (10, 3)-a, (10, 3)-c regular lattices are $n = 1.227, 1.138, 1.027, 0.952$, and 0.914 , respectively.

from edges that are broadly distributed in length (Figure 4.13A), tightly follows the scaling relation $\bar{d} \propto \rho_L^{-1/2}$ (Figure 4.17A). Across all the 240-cubes in which capillaries comprised the major vascular volume (Figure 4.3), $\bar{d} = 0.494\rho_L^{-1/2} - 3.08$, where the negative intercept is consistent with vessel radius (Figure 4.17A). This scaling relation also precisely connects the average tissue-to-vessel distance and length density previously reported for mouse vibrissa somatosensory cortex [121], where $\bar{d} = 13.3 \pm 1.2 \mu\text{m}$ and $\rho_L = 0.88 \pm 0.17 \text{ m/mm}^3$.

Similarly, the following relation accurately captures the quantitative relation between the average maximum distance between tissue and vessel wall $\overline{d_{max}}$ and vessel length density ρ_L :

$$\overline{d_{max}} = n \rho_L^{-1/2} - r_0, \quad (4.6)$$

with slope $n = 0.955 \pm 0.001$ and $r_0 = 1.85 \pm 0.03 \mu\text{m}$ (Figure 4.17B). This analysis provides a

quantitative connection between vessel-to-tissue distances and the length density of the irregular vascular networks across all regions of the brain. Given the range of slopes across all degree-3 lattices that we analyzed (Figure 4.17B and Figure 4.16), we suggest that capillary networks might be able to reduce their tissue-to-vessel distance by optimizing the structure of the network while keeping the length density unchanged.

4.6 Diffusive substrate transportation and consumption

Small substrate molecules and metabolites, like O_2 and CO_2 , respectively, transport between vessels and tissue through diffusion. How does the known heterogeneous metabolism in the brain [36, 42, 110] relate to network structure? We focus on homeostasis and assume that on the scale of hundreds of micrometers: (1) the metabolism rate and the diffusion coefficient of the substrate of interest are homogeneous throughout the tissue; (2) the concentration of the substrate in the vessel is a constant; (3) the metabolism rate is independent of substrate concentration. These assumptions allow us to summarize the properties of the substrate with a single parameter, the ratio of the diffusion coefficient, D , to the consumption rate, k , and model the concentration difference $\Delta c(\mathbf{r})$ between the vessel wall and the tissue at $\mathbf{r} = (x, y, z)$ with a Poisson equation.

$$\begin{cases} \nabla^2(\Delta c(\mathbf{r})) = \frac{k}{D}, \mathbf{r} \in \Omega_{tissue}, \\ \Delta c(\mathbf{r}) = 0, \mathbf{r} \in \partial\Omega_{vessel}. \end{cases} \quad (4.7)$$

The equilibrium substrate concentration in the tissue depends on the metabolism rate, vessel geometry, and diffusion rate. Noticing Equation 4.7 is a linear equation with a constant inhomogeneous term and a constant Dirichlet boundary condition, we first solve the following equation for the "normalized concentration difference" $u(\mathbf{r})$ for all regions in the brain:

$$\begin{cases} \nabla^2 u(\mathbf{r}) = 1, \mathbf{r} \in \Omega_{tissue}, \\ u(\mathbf{r}) = 0, \mathbf{r} \in \partial\Omega_{vessel}. \end{cases} \quad (4.8)$$

and obtain $\Delta c(\mathbf{r})$ by linearly transformation:

$$\Delta c(\mathbf{r}) = \frac{k}{D} u(\mathbf{r}) \quad (4.9)$$

In other words, under our assumption, the effects of network geometry on tissue substrate concentration can be separated from properties specific to the substrates and the metabolism rate of the brain region (k , D , and c_0).

4.6.1 Numerical solution and analyses of tissue concentration field

We numerically solved Equation 4.8 using iterative multi-scale finite difference method and analyzed the local extrema of the tissue concentration field.

The numerical solutions were obtained for the reconstructed vessel mask at different resolutions. Solutions from lower resolution were linearly interpolated to initialize the solution at higher resolutions (MATLAB implementation). The final resolution of $2 \mu m$ was a compromise between the relevant scale of capillaries, target precision, and the computation resource. At each resolution, iteration was terminated when the maximum relative update was less than 1×10^{-6} or the maximum iteration number (20000) was reached. At $2 \mu m$ voxel resolution, the solution near the vessel wall was not accurate, but our analysis was focused on the local concentration minimum. For a typical tissue volume, $u(\mathbf{r})$ calculated from $1 \mu m$ and $2 \mu m$ resolution only differed by less than 3% for tissues more than $20 \mu m$ away from the vessel wall. The maximum iteration number was set such that the characteristic diffusion distance is about an order of magnitude longer than the typical inter-capillary distance. The brain was divided into cubes of length $816 \mu m$, and each cube had $160 \mu m$ overlap with its 6-neighbors. Adiabatic boundary condition was applied on the system boundary, and the overlapping volume ($80 \mu m$ on each side) was necessary for eliminating the boundary effect. This procedure was implemented using ‘gpuArray’ in MATLAB and deployed on GPUs for parallel computation.

We used a $(50 \mu m)^3$ squared moving box to search for local normalized concentration

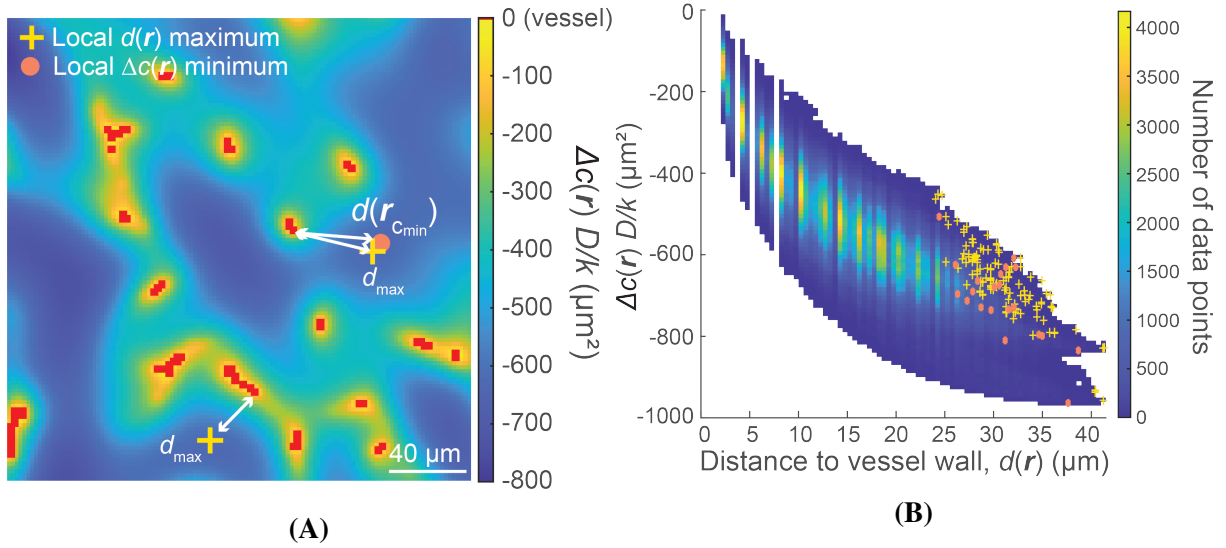


Figure 4.18. Numerical solution of steady diffusion-reaction process in tissue. (A) Numerical solution of the change in substrate concentration, $\Delta c(\mathbf{r})$, in one 240-cube. '•' marks the location of local minimum in concentration $\Delta c(\mathbf{r}_{c_{min}})$ while '+' marks the positions of local maxima in the distance map. (B) Two-dimensional histogram of the drop in substrate concentration, $\Delta c(\mathbf{r})D/k$, and vessel-to-tissue distance map, $d(\mathbf{r})$. The symbols are defined in panel A.

minima $u_{min} = \Delta c_{min}D/k$ at $\mathbf{r}_{u_{min}}$ in each 816-cube. Note that under this assumption, the locations of $u(\mathbf{r})$ local minima are exactly the same as $\Delta c(\mathbf{r})$'s (i.e., $\mathbf{r}_{u_{min}} = \mathbf{r}_{c_{min}}$). Distance transform was used to compute the distance between each local concentration minima and its nearest vessel wall $d_{c_{min}} = d(\mathbf{r}_{u_{min}})$. Results in these 816 μm -cubes were divided back into 240-cubes. For each 240-cube, we compute the average of local concentration minimum value $\overline{u_{min}}$ and the average distance between local concentration minima and their nearest vessel wall $\overline{d_{c_{min}}}$.

This procedure leads to a three-dimensional map of the change in concentration (example data in Figure 4.18A) that decreases as one moves away from a vessel until the concentration reaches a minimum where the flux of the substrate goes to zero. We label such points $\mathbf{r}(c_{min})$ (dot in Figure 4.18A).

4.6.2 Relation between concentration minima and distance map maxima

With the solutions of both $d(\mathbf{r})$ and $u(\mathbf{r})$ in 91751 240-cubes across 3 brains, we found

$$\overline{d_{c_{min}}} = 1.001 \overline{d_{max}} \approx \overline{d_{max}} \quad (4.10)$$

(Figure 4.19A). The dependence of this approximation on the window size used for searching the local extrema was further analyzed and shown in **Figure S5**. In other words, on the whole brain scale, under our assumptions, the concentration $\Delta c(\mathbf{r})$ tends to be minimized at the place where the distance $d(\mathbf{r})$ is maximized (cf •'s and +'s in Figure 4.18A and 2D histogram of Figure 4.18B), i.e., $d(\mathbf{r}(c_{min})) \approx d_{max}$. This allowed us to connect the average of the concentration minima, denoted $\overline{\Delta c(d_{c_{min}})}$ with ρ_L (Equation 4.10).

Note that though this is intuitively true for some simple system geometries, in general, it is not the case. A simple counterexample is:

$$\begin{aligned} \frac{d^2 c(r)}{dr^2} + \frac{1}{r} \frac{dc(r)}{dr} &= k, \quad r \in [R_1, R_2], \\ c(R_1) &= c(R_2) = c_0, \end{aligned} \quad (4.11)$$

whose concentration is minimized at

$$r_c = \sqrt{\frac{R_1^2 - R_2^2}{2 \log(R_1/R_2)}} \quad (4.12)$$

but the shortest distance to either cylinder is maximized at $(R_1 + R_2)/2$.

4.6.3 Analytical approximation

We then looked for an analytical formula to approximately relate $\overline{u_{min}}$ with $\overline{d_{max}}$. First, notice that generally $u(\mathbf{r})$ decreases as $d(\mathbf{r})$ increases (Figure 4.18B), but the spread of the joint distribution indicates the complexity of the numerical solution due to the vascular network geometry. Second, at local concentration minima, the concentration gradient vanishes ($\nabla u(\mathbf{r}_{u_{min}}) = 0$),

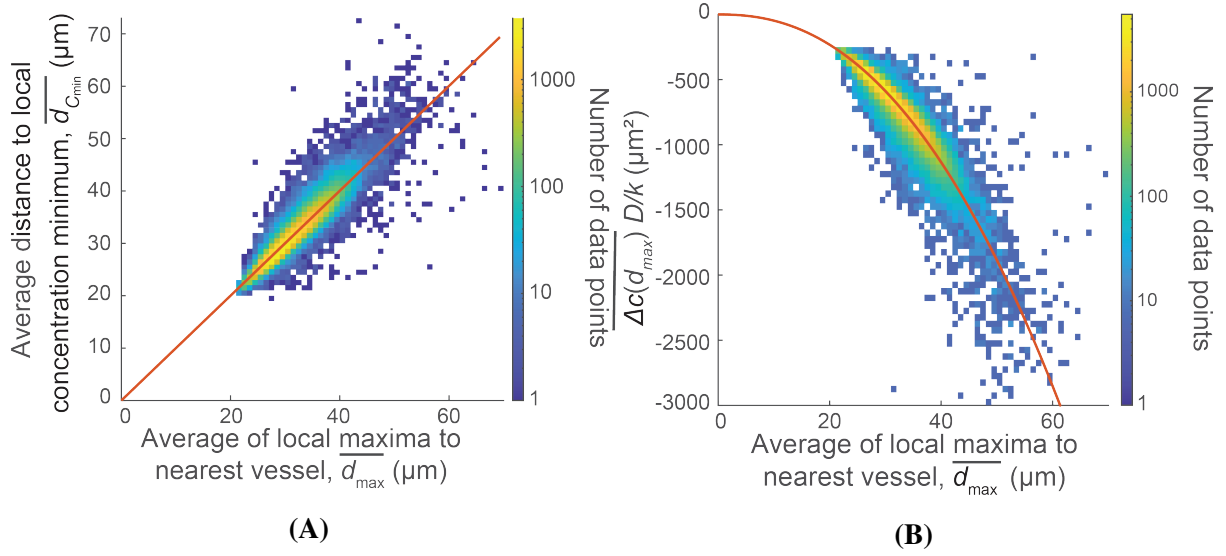


Figure 4.19. Relation between concentration gradient extrema and distance map extrema. (A) The average distance between tissue local concentration minima and their nearest vessel wall, $\overline{d_{c_{min}}}$, is statistically identical to the value of average tissue-to-vessel distance local maxima, $\overline{d_{max}}$. The slope is 1.00; $R^2 = 0.88$. (B) Best fit of the solution to the pre-scaled Poisson's equation, $\Delta c(\mathbf{r})D/k$, with pre-scaling factor $\lambda = 0.52$. The fit has $R^2 = 0.90$.

and so does the diffusion flux. Therefore, we looked for a formula that (a) describes substrate diffusion from the vessel wall, (b) has concentration minimized at $\overline{d_{c_{min}}}$, and (c) has a maximum tissue-vessel concentration difference $\overline{u_{min}}$.

The Krogh model [62] is a special case of Equation 4.7, where one infinitely long cylindrical vessel of radius r_0 presents in the tissue, and the concentration reaches a minimum level c_{min} at tissue $d_{c_{min}}$ away from the vessel wall:

$$\begin{cases} \frac{1}{r} \frac{d}{dr} (r \frac{dc}{dr}) = \frac{k}{D}, & r \in (r_0, r_{max}) \\ c(r_0) = c_0, \\ \frac{dc}{dr} |_{r=r_{max}} = 0, \end{cases} \quad (4.13)$$

where $r_{max} = d_{c_{min}} + r_0$. The solution gives the maximum substrate concentration difference

between tissue and vessel as:

$$\Delta c(r_{max}) = \frac{k}{D} \left[-\frac{r_{max}^2}{2} \log\left(\frac{r_{max}}{r_0}\right) + \frac{r_{max}^2 - r_0^2}{4} \right] = \frac{k}{D} u(r_{max}). \quad (4.14)$$

Note that Equation 4.14 takes the same form as Equation 4.9 and terms inside the squared bracket $u(r_{max})$ completely depend on the vessel geometry. The Krogh model was derived for vessels in the mussel, where capillaries are aligned along one direction with relatively uniform spacing. Brain capillary network is far less regular, and our numerical solution suggested that $u(\overline{d_{max}} + r_0)$ calculated from Equation 4.14 is about 100% higher than $\overline{u_{min}}$. Nonetheless, 4.14 provides a simple relation between concentration difference and vessel geometries for substrates transported through diffusion. To reconcile the quantitative difference, we used Equation 4.14 as a "functional form" to fit $\overline{u_{min}}$ with respect to $\overline{d_{max}}$ using linear regression, and denote the slope as λ :

$$\overline{u_{min}} = -\frac{\lambda}{2} \left[r_{max}^2 \left(\log\left(\frac{r_{max}}{r_0}\right) - \frac{1}{2} \right) + \frac{r_0^2}{2} \right], \quad (4.15)$$

where $r_{max} = \overline{d_{max}} + r_0$. This is the equation used for fitting λ in Figure 4.19B. For 91751 240-cubes across 3 brains, the fitting yielded $\lambda = 0.5185$ with $R^2 = 0.897$. Note that Equation 4.15 relates normalized concentration difference to vascular network geometrical parameters, and is independent of substrate properties and metabolism rate.

The accuracy of the fitting suggests that the functional form of the Krogh model captures most of the dependence of $\overline{u_{min}}$ on $\overline{d_{max}}$, while λ captures the shared structural properties of the vascular network across the brain. The Krogh model describes diffusion from a single vessel segment, while λ is a structural parameter that relates the single vessel solution to the vascular network solution (only at the local extrema).

4.6.4 Metabolism rate sets vascular network geometry

Finally, we apply the model across brain regions where resting-state glucose metabolism is known to vary by 3-fold [42]. Using Equation 4.9, the oxygen consumption rate k_{O_2} can be

expressed as:

$$k_{O_2} = \frac{2\alpha_{O_2}D_{O_2}}{\lambda[r_{max}^2(\log(\frac{r_{max}}{r_0}) - \frac{1}{2}) + \frac{r_0^2}{2}]} \Delta p_{O_2}, \quad (4.16)$$

where oxygen concentration has been converted to partial pressure through oxygen solubility α_{O_2} , and Δp_{O_2} is the oxygen partial pressure difference between the vessel wall and tissue oxygen partial pressure minimum.

Under homeostasis, glucose is the main energy source and the glucose consumption rate k_{glu} is related to the oxygen consumption rate k_{O_2} by $\kappa = k_{O_2}/k_{glu} = 5.65$ [80]. Furthermore, using the scaling relation between d_{max} and vessel length density Equation 4.6, we obtained a relation between vessel length density and glucose metabolism rate:

$$\begin{aligned} k_{glu} &= \frac{4\alpha_{O_2}D_{O_2}}{\kappa\lambda} \frac{\rho_L}{n^2[\log(n^2) - 1 - \log(r_0^2\rho_L)] - r_0^2\rho_L} \Delta p_{O_2} \\ &\approx \frac{4\alpha_{O_2}D_{O_2}}{\kappa\lambda} \frac{\rho_L}{n^2[\log(n^2) - 1 - \log(r_0^2\rho_L)]} \Delta p_{O_2}, \end{aligned} \quad (4.17)$$

where the average capillary radius $r_c = 2.19 \mu\text{m}$, the prefactor is $\lambda = 0.52$ (Figure 4.19B), $n = 0.955$ (Figure 4.16), and we take the limit of $r_c^2\rho_L \ll 1$, i.e., the vasculature occupies a small fraction of the brain. The substrate specific parameters are oxygen diffusion coefficient $D_{O_2} = 1.9 \mu\text{m}^2/\text{ms}$ and solubility $\alpha_{O_2} = 1.3 \mu\text{M}/\text{mmHg}$ [17]. Note that in the denominator the logarithmic term in ρ_L grows slowly, and therefore, regional glucose metabolism rate k_{glu} is approximately linear in vessel length density ρ_L if Δp_{O_2} is held a constant.

How does the scaling of observed glucose metabolism compare with our prediction? We used data measured by $[2\text{-}^{14}\text{C}]$ -glucose utilization in unstressed awake rats across 27 different brain regions [42]. These data span a factor of three in metabolic rate (Table 4.4). When matched against our measured length density for the same 27 regions, the observed glucose metabolism tracks the length density (Figure 4.20). Critically, this relation is described by Equation 4.17 using a universal drop in oxygen tension, i.e., $\Delta p_{O_2} = 15.9 \pm 0.3 \text{ mmHg}$ (mean $\pm$ SE), between the capillary wall and the locations with the lowest p_{O_2} (Figure 4.20). For fixed Δp_{O_2} , the

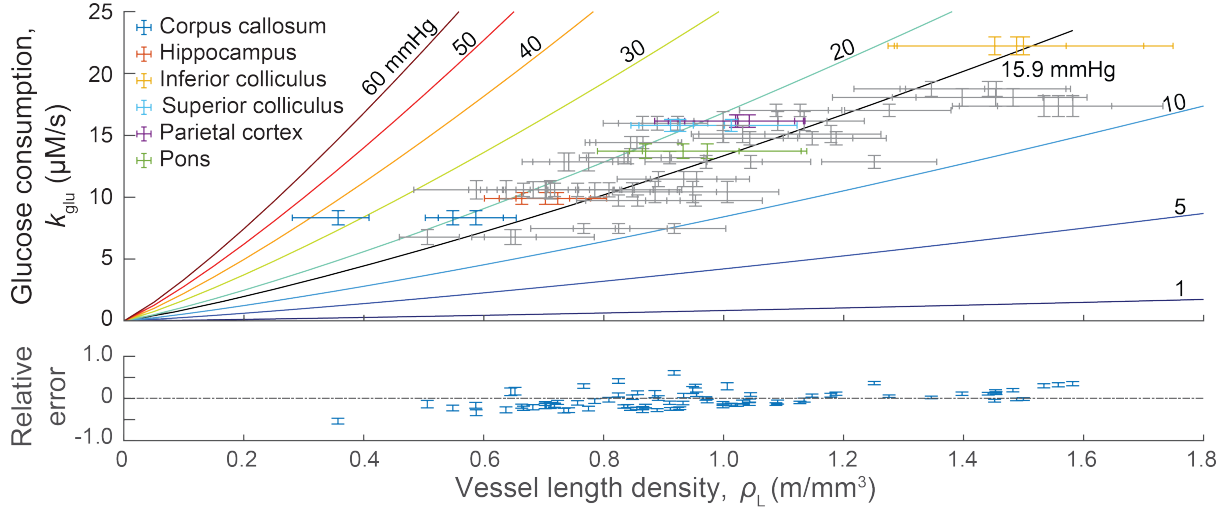


Figure 4.20. Glucose consumption rate as a function of vessel density in mice. The rate of glucose metabolism versus the vessel length density for different values of ΔpO_2 , the drop in pO_2 between the vessel wall and the minimum pO_2 in tissue. The data points are from Hawkins and colleagues [42] (converted to SI unit through the brain tissue density 1.042 g/ml [24]) and seem to maintain a roughly constant drop in pO_2 . The best fit has $R^2 = 0.756$ and an average absolute relative error of 0.17. Horizontal error bar: median and 25 - 75 percentiles of regional line density ρ_L . Vertical error bar: mean $\pm$ standard deviation.

increase of k_{glu} with ρ_L is weakly supralinear for a constant change in oxygen tension. This formulation accounts for the known correlation between metabolism rate and vessel length density [9, 130]. It is equally applicable to the diffusive removal of metabolic waste products.

4.6.5 Discussion

Organ-specific vascular patterns are highly conserved across vertebrates [66]. Thus our structural findings in rodent brains might generalize to primate vasculature. There is some limited data on the density of the microvasculature and the metabolic load in different regions of the human brain and in one region of the macaque brain (Table 4.5). We plot these data and the mouse data on a universal plot where vessel length density is normalized as the vascular fraction $r_c^2 \rho_L$ (Equation 4.17). While there is unquestioned variability, the data across three species data coalesce in the vicinity of the line for $\Delta pO_2 = 16$ mmHg (Figure 4.21). Despite the simplifying assumptions used to develop our model, the observed trend suggests a universal drop in the

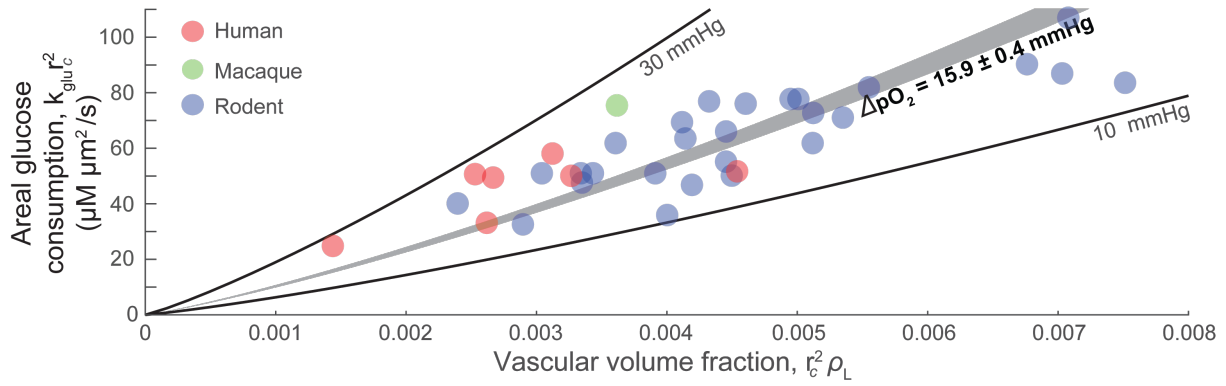


Figure 4.21. Glucose consumption rate as a function of vessel density in mammals. The areal rate of glucose metabolism versus the vascular fraction, corresponding to the vessel length density normalized by the capillary radius, i.e., $r_c^2 \rho_L$. We plot average data from three species; data from humans [15, 29, 41, 43, 50, 63, 64, 94, 118, 122] and macaque [58, 130] is listed in Table 4.5.

partial pressure of oxygen throughout mammalian brains.

How realistic is our estimate of $\Delta pO_2 \sim 16$ mmHg as the largest drop in oxygen tension within tissue in the sedentary mammal (Figure 4.20, Figure 4.21)? Hypoxic levels of pO_2 are taken to be around 10 mmHg [56], so roughly we require $pO_2 \sim 26$ mmHg near capillary walls in the sedentary state. This level is well surpassed by the experimentally measured value of $pO_2 \sim 40$ mmHg near capillary walls [78], with a similar value near penetrating arterioles [70], in the sedentary state. A more complicated situation occurs during heightened brain activity, where the rate of neuronal metabolism is believed to increase more than 1.9-fold [11], for an estimated increased drop of $\Delta pO_2 \sim 1.9 \times 16 \sim 30$ mmHg within tissue during heightened brain activity. We now require $pO_2 > 40$ mmHg near capillary walls in the active state. This can only be achieved by vasodilation and the increase in blood flow associated with the "hemodynamic response" triggered by heightened brain activity [21, 136].

Over a century ago, August Krogh reported a formula for calculating the maximum oxygen tension between capillaries and muscle cells [62]. The simplicity and validity of the so-called Krogh-Erlang model are rooted in the near-uniform orientation and regularity of the capillary network in muscles. Today, technical advances allow us to extend quantitative

physiological methods to the highly irregular capillary network in the brain. By combining biophysical analysis with numerical results, we transcended the theoretical intractability of irregular capillary networks. The surprisingly accurate scaling relation between tissue-to-vessel length and vascular length (Figure 4.17B) allowed us to connect the substrate utilization rate to the vessel length density (Figure 4.20, Figure 4.21). This explicit dependence of substrate utilization on vascular density has been anticipated by [62] and others [9, 130], but would not have been possible without accurate measurements and quantitative analysis.

4.7 Regional length distribution and network robustness

As shown previously, tissue oxygenation in general decreases as its distance to the nearest vessel. On the one hand, there is a constant background of stalls of red blood cells in a fraction of capillaries [60, 126]. This increases from about 0.5% of capillaries in normal brains to 1% to 2% in mouse models of Alzheimer’s disease [20], as well as increases during re-perfusion after ischemia [27, 28]. How does the removal of a single capillary impact the distance map? On the other hand, persistent tissue oxygen tension was believed to lead to angiogenesis, where new vessels were induced from existing vessels and guided toward the hypoxia tissue region, and therefore, the new vessels should be close to the local oxygen concentration minimum. We quantified the structural basis of robust oxygen supply and tested the hypothesis of angiogenesis by single-segment removal perturbation.

A conceptual illustration that makes use of the distance map shows the territories associated with selected edges (red solid lines in Figure 4.22A) in a two-dimensional honeycomb lattice. All points in the space that are closer to the capillary labeled C_0 than any other capillary define the territory of C_0 (gray area in Figure 4.22A), with associated distances $d(\mathbf{r})$. We quantify the impact of deleting C_0 on expanding the territory of the neighboring vessels by recomputing the shortest distance between $\mathbf{r}$ and the remaining vessels, denoted as $d_{del}(\mathbf{r})$. The territory of C_0 is redistributed solely to one of the four capillaries of branch order (BO) 1, labeled C_1 , that were

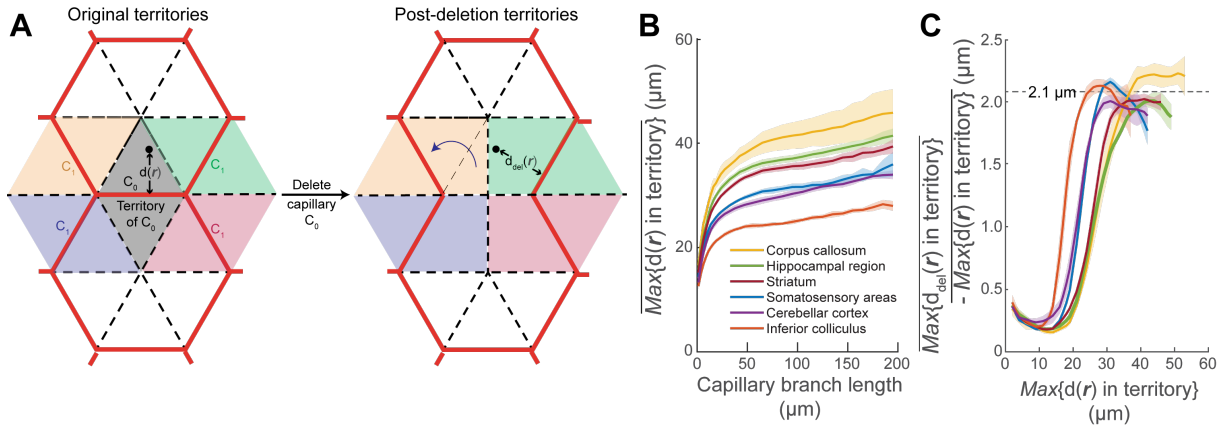


Figure 4.22. Single capillary removal effects on distance map. (A) Illustration of perturbation analysis for two unit cells of a two-dimensional honeycomb capillary lattice (red) before and after removal of one capillary, denoted C_0 . Capillaries one branch away are denoted C_1 . Tissue was assigned to the nearest capillary, i.e., gray regions to C_0 and color-coded regions to neighboring C_1 's. The scalar $d(\mathbf{r})$ is the unperturbed distance map. After the deletion of capillary C_0 , the tissue originally in the territory of C_0 was reassigned to the remaining capillaries and the perturbed distance map is $d_{\text{del}}(\mathbf{r})$. In this two-dimensional example, the territory of C_0 is completely redistributed solely to C_1 capillaries and the maximum distance to a vessel remains unchanged, i.e., $\max\{d_{\text{del}}(\mathbf{r}) \in C_0 \text{ territory}\} = \max\{d(\mathbf{r}) \in C_0 \text{ territory}\}$. (B) The maximum distance between the capillary and the furthest locations in its tissue territory. Solid lines and transparent shadow show mean and standard error as an average over capillary branches in different brain regions, the same as panel C. (C) Increase of the maximum distance between the territory of the deleted capillary and the remaining nearest vessel.

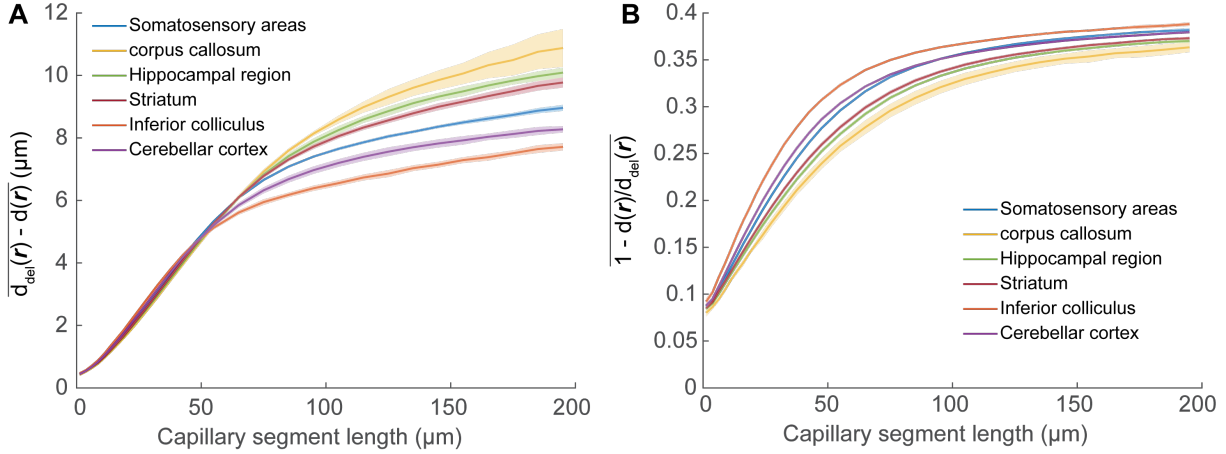


Figure 4.23. Changes in DT field after single segment removal. (A) Average increase in tissue-to-vessel distance $d_{del}(\mathbf{r}) - d(\mathbf{r})$ after single segment removal increases as capillary segment length. (B) Relative increase in tissue-to-vessel distance $1 - d(\mathbf{r})/d_{del}(\mathbf{r})$ after single segment removal increases as capillary segment length.

directly connected to C_0 .

We now estimate the impact of removing an individual capillary on the tissue-to-vessel distance map. Denote the territory of the i -th vessel as V_i . The extent of the parenchyma closest to capillary i was computed as $\max\{d(\mathbf{r}) \in V_i\}$. For each region, we computed the average overall capillaries $\overline{\max\{d(\mathbf{r}) \in V_i\}}$, which increased as a function of capillary branch length and, further, was greatest for regions with low length density, e.g., corpus callosum, compared to regions with high density (Figure 4.22B). Therefore, from the structural point of view, tissue near short vessels is less affected when blood flow in the surrounding segment is disrupted. Comparing the distance map before and after single segment removal, we found the average tissue-to-vessel distance $\overline{\Delta d^{(p)}} = \overline{d_{del}(\mathbf{r}) - d(\mathbf{r})}$ increases and capillary length. For segment lengths below about $60 \mu m$, the dependence of $\overline{\Delta d^{(p)}}$ on capillary length collapsed for all brain regions. Beyond $60 \mu m$, this dependence diverged for different brain regions, but all with smaller, yet positive slopes. Regions with denser capillary networks have smaller absolute increases yet larger relative increases in tissue-to-vessel distance (Figure 4.23). Surprisingly, the maximum increase of tissue-to-vessel distance in the affected tissue volume is modest (Figure 4.22C). Interestingly, the increase in the maximum distance calculated as an average over each region in

the brain, i.e.,

$$\overline{\Delta d_{max}^{(p)}} = \overline{\max\{d_{del}(\mathbf{r}) \in \text{territory}\} - \max\{d(\mathbf{r}) \in \text{territory}\}}$$

, is surprisingly modest (Figure 4.22C). It is negligible for territories close to a capillary and asymptotes at about $2.1 \mu\text{m}$ across all brain regions. Thus, while the loss of a significant fraction of capillaries leads to a catastrophic loss of connectivity (Figure 4.5), deletion of a single capillary appears tolerable, in agreement with experiment [87].

As an interconnected network, blocking blood flow in a single segment would affect both upstream and downstream segments. This impact weakens as the geodesic distance (or branch order, BO) to the blocked segment. We classified the neighboring segments by their branch order to the removed segment and computed the fraction of tissue volume $|V_i|$ that was re-distributed to each branch order. We defined the redistributed volume fraction (RVF) of capillary segment j as the fraction of territory perturbed by the removal of segment i that is reassigned to segment j . We define the number of neighboring segments N_{ns} of a capillary segment i as the number of capillary segments with nonzero RVF after removing segment i . For all brain regions, we found N_{ns} increase linearly for capillary segments of length greater than about $50 \mu\text{m}$ (Figure 4.24). Among these neighboring segments, segments of BO 1 primarily took over the tissue territory of the removed segment (4.25C). The neighbor segments taking up the largest fraction (about 20%–40%, 4.25B) of the impacted territory is of BO 1 for segments shorter than $40 \mu\text{m}$, while regions with denser capillary network have more higher BO neighbors taking up the largest RVF (Figure 4.25A). For all regions, the majority of the tissue territory is redistributed to the neighboring segments within 5 BO (4.25D). Combining the average reduction fraction and the redistributed volume fraction, we concluded that the oxygen supply would be probably more robust against sparse stalling in regions with higher vessel length density, for more abundant short vessel segments and higher network complexity.

Lastly, we again approximated the local oxygen concentration minimum by the local DT

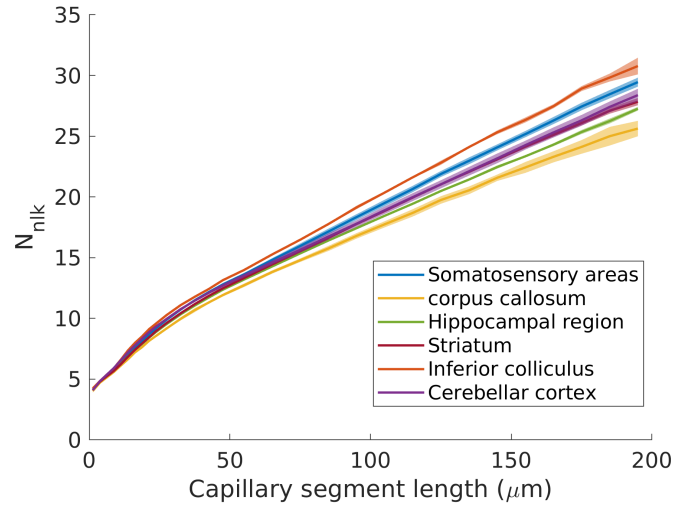


Figure 4.24. Number of neighboring segment versus capillary segment length. For all regions, the number of neighboring capillary segments increases linearly as the capillary segment length. For all regions, linear regression on segment length greater than $50 \mu m$ yielded $R^2 \geq 0.999$.

maximum, found the local DT maximum in the perturbed space, and calculated the distance between the removed segment (C_0) and the nearest local maximum (nlm):

$$dist(C_0, nlm) = \min\{dist(\mathbf{x}, \mathbf{x}_{nlm}) | \mathbf{x} \in C_0\}$$

The regional median of $dist(C_0, nlm)$ binned by segment length for each hemisphere was shown in Figure 4.26. The distance between the removed link and its nearest local DT maximum decreases as segment length. For the inferior colliculus and somatosensory cortex, more than half of the vessel segments longer than $75 \mu m$ were less than $10 \mu m$ away from the local DT maximum, and the distance asymptotically approached about $5 \mu m$. Similar decreases were observed for the striatum, hippocampus, and corpus callosum, but with higher asymptotic values. Consequently, long capillary segments tend to be close to the tissue-to-vessel distance map local maxima.

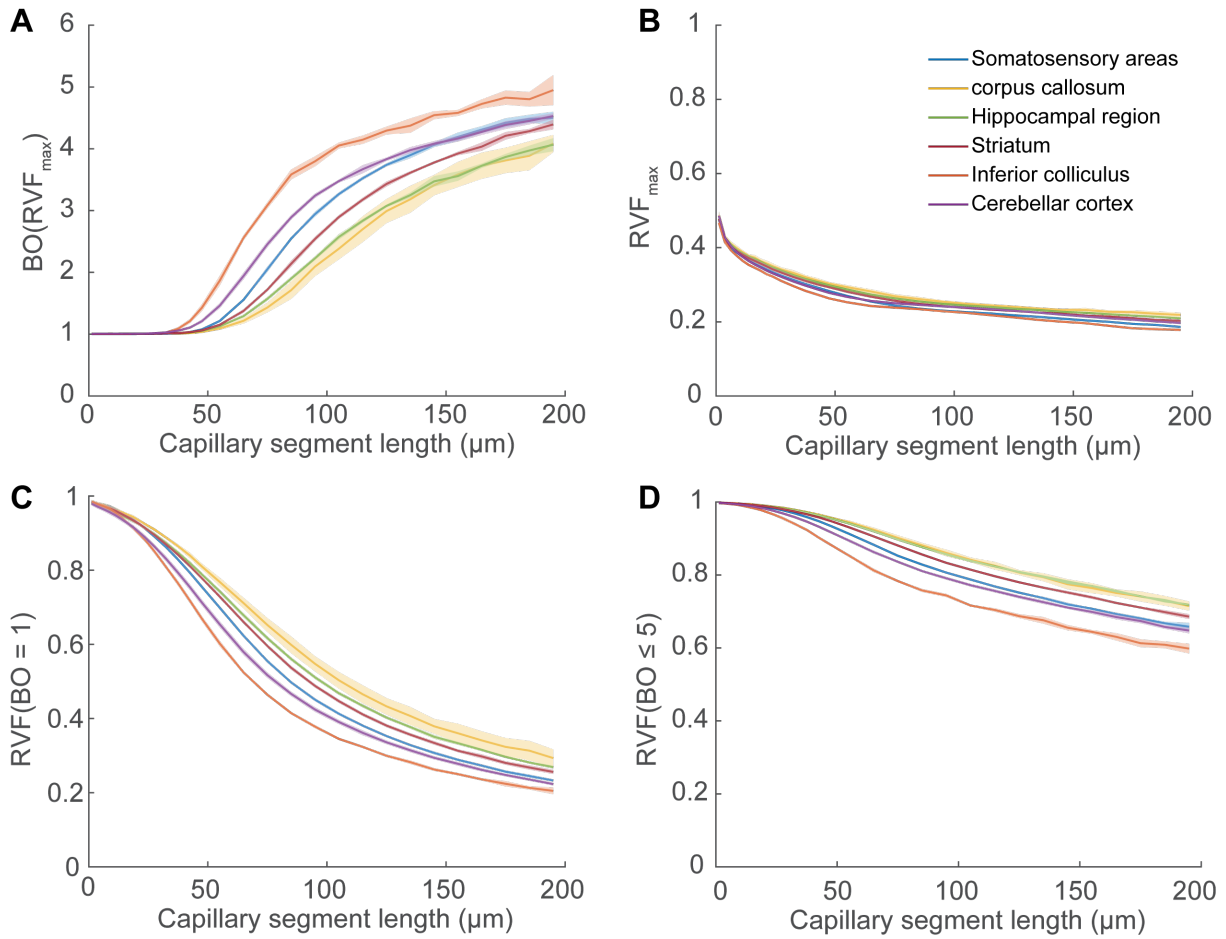


Figure 4.25. Tissue territory redistribution volume fraction. (A) Branch order of the neighboring segment with the largest RVF. (B) Maximum RVF decreases as capillary segment length. (C) The total RVF of branch-order-1 segments decreases as capillary segment length. (D) The total RVF of segments of branch-order less than 5 decreases as capillary segment length.

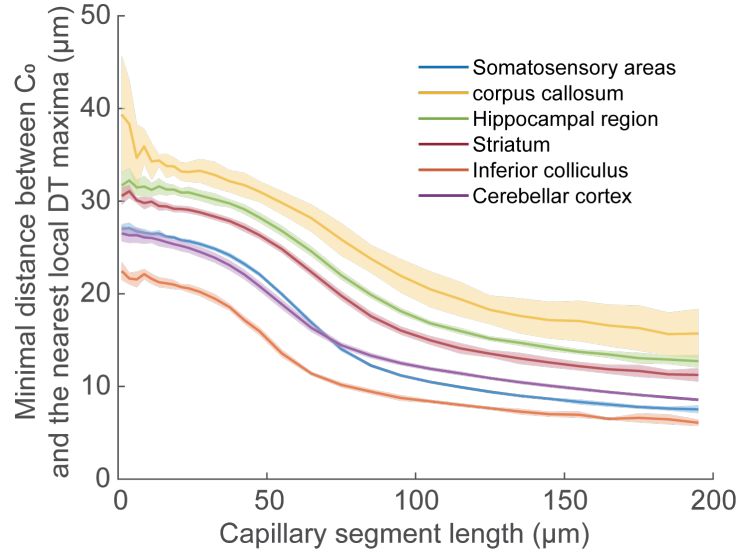


Figure 4.26. Distance between removed segment and the nearest local DT maxima. This distance decreases as capillary length.

4.8 Linear analysis of cerebral microcirculation

As the majority of brain vessels have radii on the order of $10 \mu m$ and blood flow on the order of 1 mm/s [60, 105, 53], the corresponding Reynolds number is less than 1. Therefore, to the first order approximation, cerebral blood flow can be modeled as current driven through a resistor network by voltage difference [71, 101]. Using the resistor network model, Reichold et al [101] simulates the impact of artery dilation and occlusion on flow distribution in rat cerebral vasculature. By tracking red blood cells (RBCs) as passive particles following the flow, Lorthois et al compute the changes of pressure, flow rate, and hematocrit due to global or single artery dilation in human intra-cortical vascular network [75, 74]. More advanced models account for the effect of RBCs by either approximating blood as biphasic fluid [36] or directly tracking the movement of individual RBC [76]. These models have been applied to simulate blood flow in mouse brain somatosensory cortex vascular networks, analyze hemodynamics resistance [37] and effect of capillary dilation [107], and predict cortical-depth dependent hematocrit [40, 108].

Despite several numerical studies on the effect of vasodilation on regional blood flow [101, 74, 107], the spatial specificity of the brain blood flow regulation and its relation to the structure

of the vascular network remains largely unknown. These studies analyzed numerical results, whose computational costs prohibit systematic analysis of the parameter space. We propose to use the simplest resistor network model to analyze cerebral blood flow regulation on the system level both analytically and numerically. With the simplest model, pressure at vessel bifurcation points can be solved by inverting the Laplacian matrix of the conductance-weighted vessel graph. We will focus on the network effects and ask:

- 1 How does single vessel dilation/constriction affect the blood flow in itself and in its neighboring vessels? How does its effect on the neighbors decay over topological and Euclidean distance? How does this effect depend on the vessel identity and its position in the vascular network?
- 2 With the basal flow rate computed, how do RBCs travel in the network following the flow? Given a starting vessel segment, how many downstream vessels will they travel through? What is the average transient time and travel distance? Do they have any preference? How do these properties depend on the network structure?

4.8.1 System of equations

Vessel segment resistance

We apply the phenomenological fitted model to estimate the fluid resistance of each vessel segment based on vessel geometry[97][6]. For a vessel segment of length L_{mn} and radius ρ_{mn} between node m and n , the fluid resistance r_{mn} can be computed as:

$$r_{mn} = \rho_{nm} = \alpha(r_{mn}) \frac{8\eta_{water} L_{mn}}{\pi \rho_{mn}^4}, \quad (4.18)$$

where η_{water} is the dynamic viscosity of water and its value at $37^\circ C$ is $6.913 \times 10^{-4} \text{ Pa} \cdot \text{s}$ and the modification coefficient $\alpha(\rho_{mn})$ is given by:

$$\alpha(r) = 4[1 - 0.863e^{-\frac{r}{14.3\mu m}} + 27.5e^{-\frac{r}{0.351\mu m}}]. \quad (4.19)$$

The correction coefficient reach its minimum 1.19 at $r = 2.57\mu\text{m}$ and asymptotically reach 4 as r goes to infinity.

Fix pressure boundary condition

To solve the resistor network model, external pressure or current needs to be specified at the open endpoints of the segments. These two boundary condition leads to slightly different systems of equations.

Suppose arteries and veins are pressure sources. Consider a vascular network with N_I internal nodes with unknown pressure ϕ_i , $i = 1, \dots, N_I$ and N_E external nodes with known pressure Φ_e , $e = 1, \dots, N_E$. Denote the conductance between the internal nodes i and j as c_{ij} , while the conductance between the internal node i and the external node e as $c_{ie}^{(E)}$. By the conservation of current at each internal node:

$$\sum_{j \neq i}^{N_I} c_{ij}(\phi_i - \phi_j) + \sum_e^{N_E} c_{ie}^{(E)}(\phi_i - \Phi_e) = 0, \quad (4.20)$$

which can be organized as:

$$\left(\sum_{j \neq i}^{N_I} c_{ij} + \sum_e^{N_E} c_{ie}^{(E)}\right)\phi_i - \sum_{j \neq i}^{N_I} c_{ij}\phi_j = \sum_e^{N_E} c_{ie}^{(E)}\Phi_e, \quad (4.21)$$

which can be written as $A^{(p)}\boldsymbol{\phi} = \mathbf{b}$, where $A_{ij}^{(p)} = (\sum_{j \neq i}^{N_I} c_{ij} + \sum_e^{N_E} c_{ie}^{(E)})\delta_{ij} - c_{ij}(1 - \delta_{ij})$, and p denotes for constant pressure boundary condition.

Fix flux boundary condition

Alternatively, the input flows can be fixed at the arterial and venous endpoints. Suppose the externally injected current at node i is $I_i^{(E)}$, we have:

$$\sum_{j \neq i}^{N_I} c_{ij}\phi_i - \sum_{j \neq i}^{N_I} c_{ij}\phi_j = I_i^{(E)}, \quad (4.22)$$

which can be expressed as $A\phi = \mathbf{I}^{(E)}$. A depends on the network connectivity, while is completely independent of the external source. The conductance matrix A with fix-flux boundary condition is a positive semi-definite matrix. If the entire network is single-connected, then A has one zero eigenvalue and all other eigenvalues are strictly positive, and therefore A is singular. To solve the system of equations, the Moore-Penrose pseudoinverse can be computed as:

$$A^{-1} = (A + \frac{\mathbf{e}\mathbf{e}^T}{N_I})^{-1} - \frac{\mathbf{e}\mathbf{e}^T}{N_I} \quad (4.23)$$

where $\mathbf{e} = (1, 1, 1 \dots 1)^T \in \mathbb{R}^{N_I}[10]$.

Effective network resistance

For fixed flux boundary conditions, the matrix element of the inverse matrix A^{-1} has a clean physical interpretation. The pressure at each node can be computed as $\phi_i = A_{ij}^{-1} \mathbf{I}_j^{(E)}$, i.e. the pressure at node i is the superposition of the effect of current injection into node j whose effect on i is determined by the value of the i -th term of the j -th "propagator" (j -th column of A^{-1}).

Consider external current I and $-I$ are injected to node i and j , respectively, while all the other nodes do not receive external current. Denote $\Delta(i, j)_m = \delta_{mi} - \delta_{mj}$ be a column vector with the i -th element 1, j -th element -1, and the rest 0. $\mathbf{I}^{(E)} = I^{(E)} \Delta(i, j)$. The effective network resistance between node i and j can be computed as:

$$R_{ij} = \frac{\phi_i - \phi_j}{I^{(E)}} = \frac{\Delta^T(i, j) A^{-1} \mathbf{I}^{(E)} \Delta(i, j)}{I^{(E)}} = \Delta^T(i, j) A^{-1} \Delta(i, j) \quad (4.24)$$

The induced "displacement current" in segment (m, n) due to current injection in segment (α, β) can be computed as:

$$i_{mn} = c_{mn} \Delta^T(m, n) A^{-1} \Delta(\alpha, \beta) I^{(E)} \equiv f_{\alpha\beta}^{mn} I^{(E)}, \quad (4.25)$$

where $f_{\alpha\beta}^{mn}$ is the fractional current response in segment (m, n) in response to current injection in segment (α, β) and therefore $f_{\alpha\beta}^{mn} \in [-1, 1]$. Effectively, the network around segment (α, β) can be simplified to two parallel resistors with overall resistance $R_{\alpha\beta}$ and one of the resistors has conductance $c_{\alpha\beta}$. The total resistance of all the remaining paths connecting node α and β is then $R_{\alpha\beta}/(1 - f_{\alpha\beta}^{\alpha\beta})$.

For a monopole current injection, current flows to infinity, and by the conservation of charger, $I_{mn} \propto r_{mn}^{-2}$ in three-dimensional space, where r_{mn} is some distance between the injected site (α, β) and segment (m, n) . For dipole current injection, $I_{mn} \propto r_{mn}^{-3}$ [34]. For a homogeneous network, the number of edges at distance r from an edge increases as $4\pi r^2$. Therefore, the probability density function of the displacement currents, $P(i_{mn}) \propto (i_{mn})^{-1/3} |\frac{dr}{di_{mn}}| \propto I_{mn}^{-2}$

In contrast, the interpretation of the fixed voltage boundary condition is less clear. The structure of $A^{(p)}$ differs from A by the conductance of the segments to the external pressure sources. $A^{(p)}$ includes the position of the external sources but is independent of the specific pressure. In other words, switching an artery to a vein does not change $A^{(p)}$, given the conductance unchanged. Considering nodes i and j being connected to external pressure source ϕ_0 and $-\phi_0$, respectively, through edges with conductance c_0 , the resulting source term has element $\mathbf{b} = c_0\phi_0\Delta(i, j)$, and the pressure difference across segment (i, j) is

$$\phi_i - \phi_j = \Delta^T(i, j)(A^{(p)})^{-1}\mathbf{b} = c_0\phi_0\Delta^T(i, j)(A^{(p)})^{-1}\Delta(i, j) \quad (4.26)$$

It is not clear how does ϕ depend on the pressure boundary condition ϕ_0 , and obviously $(A^{(p)})^{-1} \neq A^{-1}$.

Annotated vascular network and numerical solution

We manually annotated one $(1.072 \text{ mm})^3$ volume of the reconstructed vascular network near the surface of the somatosensory cortex (Figure 4.27). Arteries and veins were identified by back tracing to the major cerebral vessels in the downsampled whole brain images. This

local network contains 18973 vessel segments and 16418 branch points. We analyzed the properties of the perturbation properties of the network under fixed flux boundary conditions using Equation 4.23. In practice, the flow rate in the boundary vessel segment is hard to specify due to the lack of measurement. Therefore, we apply a fixed pressure boundary condition to the open ends of the arteries and veins to be 50 mmHg and 0 mmHg, respectively[6].

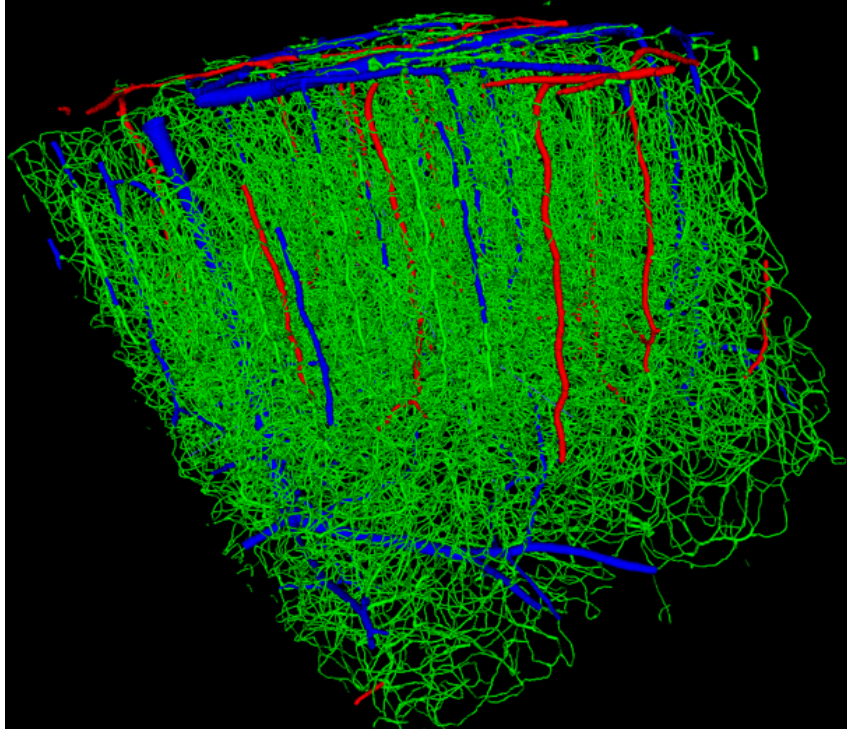


Figure 4.27. Rendering of the cortical vascular network in $(1.072mm)^3$ Arteries are in red, veins are in blue, capillaries are in green.

4.8.2 Single-vessel perturbation: self-regulation

Consider a vessel segment between two internal nodes α and β . The flow through the segment, $I_{\alpha\beta}$, is given by:

$$I_{\alpha\beta} = c_{\alpha\beta}(\phi_{\alpha} - \phi_{\beta}) = c_{\alpha\beta}\Delta^T(\alpha, \beta)\phi, \quad (4.27)$$

Suppose that this vessel segment dilates and its conductance is increased by ε , then A gets a rank-1 modification:

$$A' = A + \varepsilon \mathbf{\Delta}(\alpha, \beta) \mathbf{\Delta}^T(\alpha, \beta) \quad (4.28)$$

and the inverse of A' is given by the Sherman-Morrison formula:

$$(A')^{-1} = A^{-1} - \varepsilon \frac{A^{-1} \mathbf{\Delta}(\alpha, \beta) \mathbf{\Delta}^T(\alpha, \beta) A^{-1}}{1 + \varepsilon \mathbf{\Delta}^T(\alpha, \beta) A^{-1} \mathbf{\Delta}(\alpha, \beta)} \quad (4.29)$$

The resulting flow after dilation, $I'_{\alpha\beta}$, is given by:

$$I'_{\alpha\beta} = (c_{\alpha\beta} + \varepsilon) \mathbf{\Delta}^T(\alpha, \beta) (A')^{-1} \mathbf{b} = \frac{1 + \varepsilon/c_{\alpha\beta}}{1 + (\varepsilon/c_{\alpha\beta}) f_{\alpha\beta}^{\alpha\beta}} I_{\alpha\beta} \quad (4.30)$$

and the normalized flow change is:

$$\frac{\Delta I_{\alpha\beta}}{I_{\alpha\beta}} = \frac{1 - f_{\alpha\beta}^{\alpha\beta}}{1 + \hat{\varepsilon}_{\alpha\beta} f_{\alpha\beta}^{\alpha\beta}} \hat{\varepsilon}_{\alpha\beta} \quad (4.31)$$

where $\hat{\varepsilon}_{\alpha\beta} = \varepsilon/c_{\alpha\beta} \in [-1, \infty)$ is the fractional conductance changes in edge (α, β) . As the effective network resistance $R_{\alpha\beta}$ is non-greater than the segment resistance $r_{\alpha\beta} = c_{\alpha\beta}^{-1}$, $f_{\alpha\beta}^{\alpha\beta} = c_{\alpha\beta} R_{\alpha\beta} \in [0, 1]$. Therefore, single vessel dilation always leads to an increase in flow in itself. For $\hat{\varepsilon}_{\alpha\beta} = -1$, the segment is completely blocked, and $I'_{\alpha\beta} = 0$ as expected. However, single vessel dilation or restriction would never lead to the reverse of flow direction.

We computed the self-regulation factor $f_{\alpha\beta}^{\alpha\beta}$ for all the vessel segments in the simulation volume according to Equation 4.31. For cortical microvessels, $f_{\alpha\beta}^{\alpha\beta}$ decreases as the segment length (Figure 4.28A), implying fractional conductance increase in longer segments leads larger increase of flow in the segment. A 100 μm long capillary is expected to increase blood flow by about 20% if its radius is increased by 10%, which is quantitatively consistent with the result obtained by more sophisticated numerical result[107].

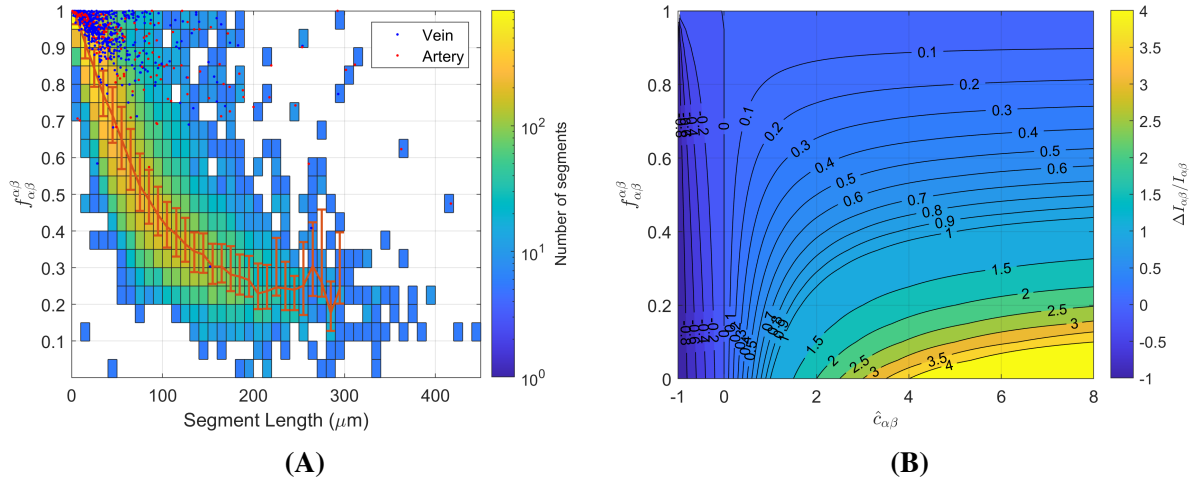


Figure 4.28. Self-regulation flexibility of blood flow. (A) Joint distribution of $f_{\alpha\beta}^{\alpha\beta}$ and vessel segment length. Red crosses indicate arteries. (B) Contour plot of the fractional flow change $\Delta I_{\alpha\beta}/I_{\alpha\beta}$ with respect to $f_{\alpha\beta}^{\alpha\beta}$ and fractional conductance change $\hat{e}_{\alpha\beta}$ in Equation 4.31.

Interestingly, most of the arteries and veins have $f_{\alpha\beta}^{\alpha\beta} > 0.85$. To understand the effect of conductance changes in different vessels, we examine the contour plot of the fractional flow change in the controlling vessel segment $\Delta I_{\alpha\beta}/I_{\alpha\beta}$ with respect to the self-regulation factor and the fractional conductance change in Figure 4.28B. As arteries have $f_{\alpha\beta}^{\alpha\beta}$ close to 1, modest vasodilation or constriction does not change the flow in itself significantly. With $f_{\alpha\beta}^{\alpha\beta} = 0.9$, the flow only increases less than 10% when the segment conductance is increased by 3 times. This explains the modest change of blood flow under strong artery dilation and the potential importance of coordinated vasodilation across the entire artery tree for local blood flow regulation. Moreover, veins have $f_{\alpha\beta}^{\alpha\beta}$ closer to 1 than arteries, suggesting that the vascular network on the venous side has a weaker ability to modulate the flow in itself - also consistent with the lack of contractile cells in the venous vessel wall (Figure 4.29A). Do the arteries with a smaller radius, i.e., arterioles, have smaller $f_{\alpha\beta}^{\alpha\beta}$? From the joint distribution of arterial self-modulation factor and the radius, the answer is not clear (Figure 4.29B).

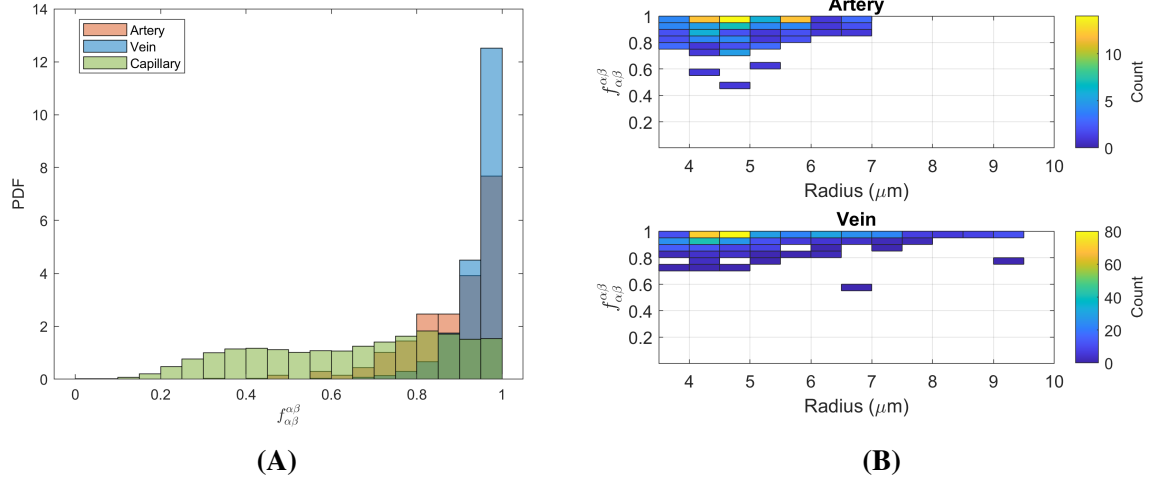


Figure 4.29. Vessel segment auto-regulation factor $f_{\alpha\beta}^{\alpha\beta}$. (A) $f_{\alpha\beta}^{\alpha\beta}$ for arteries, veins and capillaries. (B) $f_{\alpha\beta}^{\alpha\beta}$ - vessel radius joint distribution

Normalized flux and flow speed response to radius

One experimental measurable relation is the normalized flux change $\Delta I_{\alpha\beta}/I_{\alpha\beta}$ versus the normalized radius change. If we neglect the dependence of effective viscosity on vessel radius, then vessel conductance $c \propto \rho^4$ and $\hat{\epsilon}_{\alpha\beta} = [(\hat{\rho}_{\alpha\beta} + 1)^4 - 1]$, where $\hat{\rho}_{\alpha\beta} = \Delta\rho_{\alpha\beta}/\rho_{\alpha\beta}$ is the normalized radius change. Therefore

$$\frac{\Delta I_{\alpha\beta}}{I_{\alpha\beta}} = \frac{(1 - f_{\alpha\beta}^{\alpha\beta})[(\hat{\rho}_{\alpha\beta} + 1)^4 - 1]}{1 + f_{\alpha\beta}^{\alpha\beta}[(\hat{\rho}_{\alpha\beta} + 1)^4 - 1]} \quad (4.32)$$

For linear response, the slope k_I at $\hat{\rho} = 0$ is related to $f_{\alpha\beta}^{\alpha\beta}$ through $f_{\alpha\beta}^{\alpha\beta} = 1 - k_I/4$.

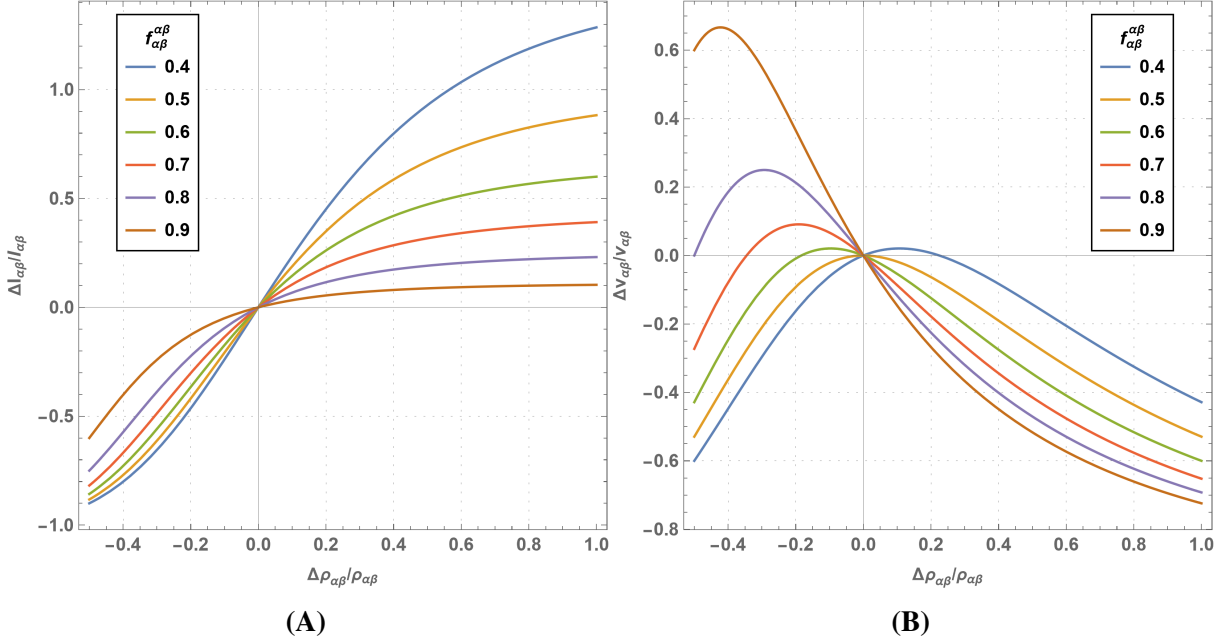


Figure 4.30. Normalized flux and flow speed response to radius. (A) Normalized flux change in a vessel in response to its radius change. (B) Normalized flow speed change in a vessel in response to its radius change.

Similarly, flow speed $v_{\alpha\beta} = I_{\alpha\beta} / (\pi \rho_{\alpha\beta}^2)$, the speed after radius modulation, v' , is related to the original speed through:

$$v'_{\alpha\beta} = \frac{\sqrt{1 + \hat{\epsilon}_{\alpha\beta}}}{1 + \hat{\epsilon}_{\alpha\beta} f_{\alpha\beta}^{\alpha\beta}} v_{\alpha\beta} \quad (4.33)$$

Taking derivation with respect to $\hat{\epsilon}$ gives:

$$\frac{dv'_{\alpha\beta}}{d\hat{\epsilon}_{\alpha\beta}} = \frac{(1 - 2f_{\alpha\beta}^{\alpha\beta}) - f_{\alpha\beta}^{\alpha\beta} \hat{\epsilon}_{\alpha\beta}}{2\sqrt{1 + \hat{\epsilon}_{\alpha\beta}}(1 + f_{\alpha\beta}^{\alpha\beta} \hat{\epsilon}_{\alpha\beta})^2} v_{\alpha\beta} \quad (4.34)$$

For $f_{\alpha\beta}^{\alpha\beta} > 1/2$, vasodilation always leads to a decrease in flow speed and therefore increase of single vessel red blood cell transient time. For $f_{\alpha\beta}^{\alpha\beta} < 1/2$, weak dilation ($0 < \hat{\epsilon}_{\alpha\beta} < (1 - 2f_{\alpha\beta}^{\alpha\beta}) / f_{\alpha\beta}^{\alpha\beta}$) leads to increase of flow speed, while stronger dilation leads to decrease of flow speed Figure 4.30B. At $\rho = 0$, the slope of normalized velocity change k_v is related to $f_{\alpha\beta}^{\alpha\beta}$ through $f_{\alpha\beta}^{\alpha\beta} = 1/2 - k_v/4$. Therefore, dilation of most arteries, veins, and short microvessels

(e.g., $< 75\mu m$) would lead to a decrease in flow speed. Dilation of long capillaries would increase the flow speed and then decrease the speed. Decreasing flow speed is expected to increase the Red blood cell transient time and therefore increase oxygen offload from the dilating vessel segment.

4.8.3 Single vessel perturbation: neighbors

Now let's consider the effect of dilation of a vessel segment on another segment between internal nodes m and n . Through similar derivation, we have:

$$I'_{mn} = c_{mn} \Delta^T(m, n) (A')^{-1} \mathbf{b} = \left[1 - \frac{f_{\alpha\beta}^{mn}}{1 + \hat{\epsilon}_{\alpha\beta} f_{\alpha\beta}^{\alpha\beta}} \frac{I_{\alpha\beta}}{I_{mn}} \hat{\epsilon}_{\alpha\beta} \right] I_{mn} \quad (4.35)$$

and

$$\Delta I_{mn} = - \frac{\hat{\epsilon}_{\alpha\beta} f_{\alpha\beta}^{mn}}{1 + \hat{\epsilon}_{\alpha\beta} f_{\alpha\beta}^{\alpha\beta}} I_{\alpha\beta}. \quad (4.36)$$

Therefore, the change of flow in the neighboring vessels is proportional to the basal flow rate in the controlling vessel, the prefactor depending on network structure ($f_{\alpha\beta}^{mn}$ and $f_{\alpha\beta}^{\alpha\beta}$) and fractional conductance change $\hat{\epsilon}_{\alpha\beta}$. For effective regulation of blood flow, the actively controlled segment should have a high flow rate. The spatial extent of the significantly affected vessel segments depends on the decay of $f_{\alpha\beta}^{mn}$ over Euclidean or geodesic distance.

The change of flow in segment (m, n) is related to the change of flow in the controlling segment (α, β) :

$$\frac{\Delta I_{mn}}{\Delta I_{\alpha\beta}} = - \frac{f_{\alpha\beta}^{mn}}{1 - f_{\alpha\beta}^{\alpha\beta}}, \quad (4.37)$$

which is completely determined by the network structure, independent of the strength of the perturbation $\epsilon_{\alpha\beta}$. Similarly, for two segments (m, n) and (p, q) responding to the controlling segment (α, β) , their changes in flow is related by:

$$\frac{\Delta I_{mn}}{\Delta I_{pq}} = \frac{f_{\alpha\beta}^{mn}}{f_{\alpha\beta}^{pq}} \quad (4.38)$$

which is also independent of the perturbation strength. If we further assume the changes in the radius of segment (m, n) and (p, q) are negligible, then the ratio of the change in flow speed is given by $\Delta v_{mn}/\Delta v_{pq} = f_{\alpha\beta}^{mn}/f_{\alpha\beta}^{pq}$.

The normalized fractional flow change in Equation 4.35 can be re-grouped as follows:

$$\frac{\Delta I_{mn}}{I_{mn}} = -\frac{\hat{\varepsilon}_{\alpha\beta}}{1 + \hat{\varepsilon}_{\alpha\beta} f_{\alpha\beta}^{mn}} \frac{I_{\alpha\beta}}{I_{mn}} f_{\alpha\beta}^{mn} \quad (4.39)$$

The first factor accounts for the network structure of the controlling vessel and its change of conductance, as shown in the counterplot (Figure 4.31A). As most of the arteries have $f_{\alpha\beta}^{\alpha\beta} > 0.8$ (Figure 4.29), the value of the first factor is about 1 even when the vessel conductance increases 8 times. The second factor is the ratio of the basal blood flow in the two vessels of interest. Consider (α, β) being an artery while (m, n) being a capillary, $I_{\alpha\beta}/I_{mn} = (v_{\alpha\beta}/v_{mn})(\rho_{\alpha\beta}/\rho_{mn})^2$. In mouse somatosensory cortex, arteries have a blood flow speed of about 10mm/s and radius $5\mu\text{m}$, while capillaries have a flow speed of about 1mm/s and radius $2.5\mu\text{m}$ [60, 53]. Thus, the typical ratio of $I_{\alpha\beta}/I_{mn}$ is about 40. If we want a 5% flow change in a capillary (m, n) , $f_{\alpha\beta}^{mn} > 10^{-3}$, which is determined by the network structure and the distance between (m, n) and (α, β) .

To examine the spatial dependence of $f_{\alpha\beta}^{mn}$, for vessel segment (α, β) we find the vessels (m, n) in the network with $f_{\alpha\beta}^{mn} > 0.001$, and compute the Euclidean distance between the middle points of (α, β) and (m, n) . We then bin the spatial separation by the $f_{\alpha\beta}^{mn}$ in log scale and compute the median and 25-/75- percentiles of the distance in each bin. Both the joint distributions and the $f_{\alpha\beta}^{mn}$ -binned statistics are plotted for capillaries, arteries, and veins, as shown in Figure 4.32.

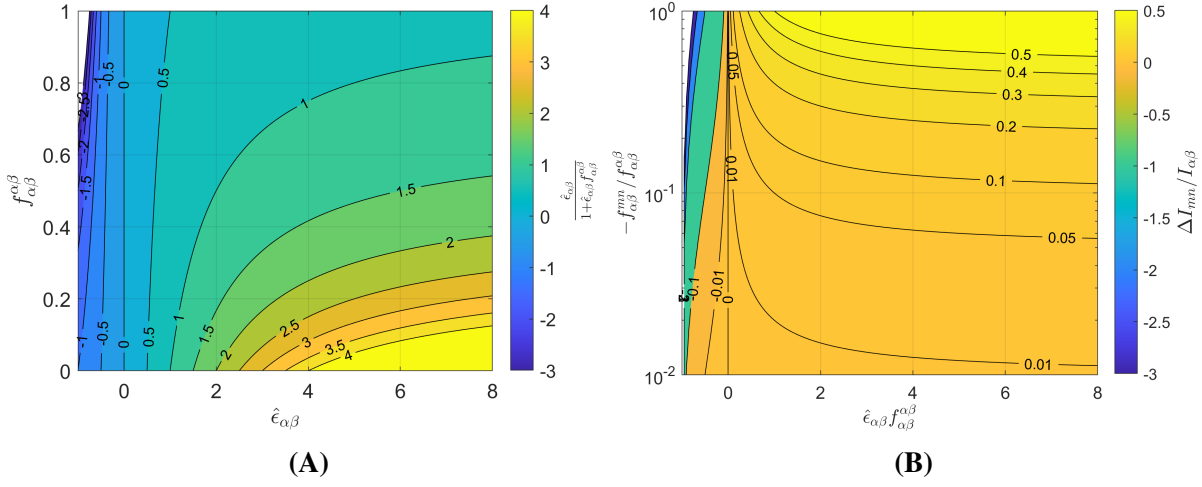


Figure 4.31. Counter plots for examining Equation 4.39. (A) Counter plots for the first factor in Equation 4.39 with respect to the normalized changes of conductance $\hat{\epsilon}_{\alpha\beta}$ and the auto-regulation factor $f_{\alpha\beta}^{\alpha\beta}$ (B) Contour plot of $\Delta I_{mn}/I_{\alpha\beta}$ with respect to $\hat{\epsilon}_{\alpha\beta} f_{\alpha\beta}^{\alpha\beta}$ and $f_{\alpha\beta}^{mn}/f_{\alpha\beta}^{\alpha\beta}$

For all types of vessels, the effect of conductance change in segment (α, β) on the neighboring segments decreases as the spatial separation increases. Given $f_{\alpha\beta}^{mn}$, arteries appear to be able to affect the flow in its neighboring vessels, presumably mostly capillaries, further away (Figure 4.32). The statistical significance should be checked later.

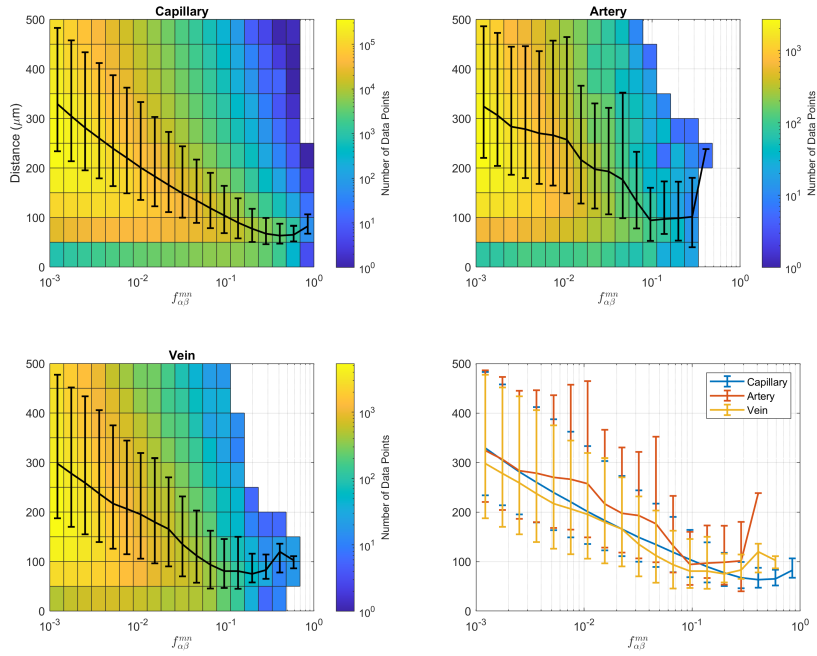


Figure 4.32. Distance and $f_{\alpha\beta}^{mn}$ between different pairs of vessels of different types

Two vessels perturbation

Consider two segments (α, β) and (μ, ν) changing their conductance by ε_1 and ε_2 respectively. For simplicity, denote $\mathbf{u} = \mathbf{\Delta}(\alpha, \beta)$ and $\mathbf{v} = \mathbf{\Delta}(\mu, \nu)$, $B_{\mu\nu}^{\alpha\beta} = (A^{-1})_{\alpha\mu} + (A^{-1})_{\beta\nu} -$

$(A^{-1})_{\alpha v} - (A^{-1})_{\beta v}$. The modification to A^{-1} can be calculated iteratively:

$$\begin{aligned}
& (A + \varepsilon_1 \mathbf{u}\mathbf{u}^T + \varepsilon_2 \mathbf{v}\mathbf{v}^T)^{-1} \\
&= A^{-1} - \varepsilon_1 \frac{A^{-1} \mathbf{u}\mathbf{u}^T A^{-1}}{1 + \varepsilon_1 B_{\alpha\beta}^{\alpha\beta}} - \varepsilon_2 \frac{(A^{-1} - \varepsilon_1 \frac{A^{-1} \mathbf{u}\mathbf{u}^T A^{-1}}{1 + \varepsilon_1 B_{\alpha\beta}^{\alpha\beta}}) \mathbf{v}\mathbf{v}^T (A^{-1} - \varepsilon_1 \frac{A^{-1} \mathbf{u}\mathbf{u}^T A^{-1}}{1 + \varepsilon_1 B_{\alpha\beta}^{\alpha\beta}})}{1 + \varepsilon_1 \mathbf{v}^T (A^{-1} - \varepsilon_1 \frac{A^{-1} \mathbf{u}\mathbf{u}^T A^{-1}}{1 + \varepsilon_1 B_{\alpha\beta}^{\alpha\beta}}) \mathbf{v}} \\
&= A^{-1} - \varepsilon_1 \frac{A^{-1} \mathbf{u}\mathbf{u}^T A^{-1}}{1 + \varepsilon_1 B_{\alpha\beta}^{\alpha\beta}} - \varepsilon_2 \frac{A^{-1} \mathbf{v}\mathbf{v}^T A^{-1}}{1 + \varepsilon_2 B_{\mu\nu}^{\mu\nu}} \\
&\quad + \frac{\varepsilon_1 \varepsilon_2 B_{\mu\nu}^{\alpha\beta}}{(1 + \varepsilon_1 B_{\alpha\beta}^{\alpha\beta})(1 + \varepsilon_2 B_{\mu\nu}^{\mu\nu})} A^{-1} (\mathbf{u}\mathbf{v}^T + \mathbf{v}\mathbf{u}^T) A^{-1} + \mathcal{O}((\sqrt{\varepsilon_1 \varepsilon_2} B_{\mu\nu}^{\alpha\beta})^2) \\
&= A^{-1} - \varepsilon_1 \frac{A^{-1} \mathbf{u}\mathbf{u}^T A^{-1}}{1 + \hat{\varepsilon}_{\alpha\beta} f_{\alpha\beta}^{\alpha\beta}} - \varepsilon_2 \frac{A^{-1} \mathbf{v}\mathbf{v}^T A^{-1}}{1 + \hat{\varepsilon}_{\mu\nu} f_{\mu\nu}^{\mu\nu}} \\
&\quad + \frac{A^{-1} (\varepsilon_{\alpha\beta} \hat{\varepsilon}_{\mu\nu} f_{\alpha\beta}^{\mu\nu} \mathbf{u}\mathbf{v}^T + \varepsilon_{\mu\nu} \hat{\varepsilon}_{\alpha\beta} f_{\mu\nu}^{\alpha\beta} \mathbf{v}\mathbf{u}^T) A^{-1}}{(1 + \hat{\varepsilon}_{\alpha\beta} f_{\alpha\beta}^{\alpha\beta})(1 + \hat{\varepsilon}_{\mu\nu} f_{\mu\nu}^{\mu\nu})} + \mathcal{O}((f_{\mu\nu}^{\alpha\beta})^2)
\end{aligned} \tag{4.40}$$

The change of flow in segment (m, n) ($(m, n) \neq (\alpha, \beta), (\mu, \nu)$) is then:

$$\Delta I_{mn} \approx -\frac{\hat{\varepsilon}_{\alpha\beta} f_{\alpha\beta}^{mn}}{1 + \hat{\varepsilon}_{\alpha\beta} f_{\alpha\beta}^{\alpha\beta}} I_{\alpha\beta} - \frac{\hat{\varepsilon}_{\mu\nu} f_{\mu\nu}^{mn}}{1 + \hat{\varepsilon}_{\mu\nu} f_{\mu\nu}^{\mu\nu}} I_{\mu\nu} + \frac{\hat{\varepsilon}_{\alpha\beta} \hat{\varepsilon}_{\mu\nu} (f_{\mu\nu}^{mn} f_{\alpha\beta}^{\mu\nu} I_{\alpha\beta} + f_{\alpha\beta}^{mn} f_{\mu\nu}^{\alpha\beta} I_{\mu\nu})}{(1 + \hat{\varepsilon}_{\alpha\beta} f_{\alpha\beta}^{\alpha\beta})(1 + \hat{\varepsilon}_{\mu\nu} f_{\mu\nu}^{\mu\nu})} \tag{4.41}$$

Notice that $f_{\mu\nu}^{\alpha\beta}$ is the fraction of flow segment (α, β) receives for external current injected into segment (μ, ν) and therefore $f_{\mu\nu}^{\alpha\beta} \in [0, 1]$ and decreases as the spacing between (α, β) and (μ, ν) increases. The first-order correction term is significant when (α, β) and (μ, ν) are adjacent edges in the graph, suggesting the cooperative effect of hierarchical vessel radius modulation.

4.8.4 Downstream flow distribution

Given a boundary condition, using the method described above, we can compute the flow in all the vessel segments numerically. The flow in all the vessels can be used to construct a directed graph, whose connectivity matrix $C_{ij} = 1$ if segment j is the direct downstream of

segment i . The fractional flow from segment i to segment j in one step is then:

$$Q_{ij} = \frac{I_j}{\sum_k C_{ik} I_k} = \frac{C_{ij} I_j}{I_i} \quad (4.42)$$

and the total fractional flow from segment i to segment j is:

$$Q_F = \sum_{n=0}^{\infty} Q^n = (1 - Q)^{-1} \quad (4.43)$$

For numerical implementation, $Q^{(1)}$ is a sparse matrix and the vascular graph might contain more than one connected component. Therefore, it would be more efficient to compute Q_F by summing the exponent of a sparse matrix $Q^{(1)}$ to the order when all the matrix elements vanish.

For segment j , the fraction of its flow from segment i , $[Q_B]_{ij}$ is given by:

$$[Q_B]_{ij} = \frac{I_i [Q_F]_{ij}}{I_j} \quad (4.44)$$

Average trajectory statistics

For segment i with length l_i and flow speed v_i , the segment transient time is $\tau_i = l_i/v_i$. We are interested in the average transient time for a passive particle that follows the flow to move from segment α to segment β . Consider an initial distribution $[\mathbf{p}(0)]_i = \delta_{\alpha i}$ and the initial cumulative transient time $[\mathbf{t}(0)]_i = [\mathbf{p}(0)]_i \tau_i$, the distribution after one step of evolution is $\mathbf{p}(1)^T = \mathbf{p}(0)^T Q$ and the cumulative segment transient time is the weighted sum of the cumulative transient time in the upstream segments, plus the product of the flow fraction and the segment transient time:

$$\mathbf{t}(1)^T = \mathbf{t}(0)^T Q + \mathbf{p}(1)^T \Lambda \quad (4.45)$$

where $\Lambda = \text{diag}(\tau_1, \tau_2, \dots, \tau_N)$. This process can be iterative until all elements of $\mathbf{t}(n)$ vanish.

The average transient time from segment α to β is then:

$$T_{\alpha\beta} = \frac{\sum_n [\mathbf{t}(n)]_\beta}{\sum_n [\mathbf{p}(n)]_\beta} \quad (4.46)$$

To calculate the elements of $\mathbf{T}$ simultaneously, we define:

$$\begin{aligned} S_1 &= \Lambda Q + Q \Lambda \\ S_n &= S_{n-1} Q + Q^n \Lambda, \quad n > 1 \end{aligned} \quad (4.47)$$

then

$$T_{\alpha\beta} = \frac{\sum_n [S_n]_{\alpha\beta}}{[Q_F]_{\alpha\beta}} \quad (4.48)$$

can be computed as matrix element-wise summation and division.

By changing the construction of matrix Λ , the procedure described above can also be used to compute the average path length between any pairs of segments. For $\Lambda = \mathbb{I}$, we have $S_n = (n+1)Q^n$ and

$$S = \sum_n (n+1)Q^n = (\mathbb{I} - Q)^{-2} = Q_F^2. \quad (4.49)$$

Therefore the average branch order from segment α to segment β is

$$T_{\alpha\beta} = \frac{[Q_F^2]_{\alpha\beta}}{[Q_F]_{\alpha\beta}} \quad (4.50)$$

Prior numerical simulation has observed that red blood cells tend to enter and exit the capillary bed at a similar cortical depth [106]. To see if this laminar blood flow pattern is captured by the linear resistor network model, we define the capillaries that are directly connected to arteries (veins) as the first arterial (venous) order capillary, compute their distance to the cortical surface using the distance transform of the whole brain mask, and compute the fraction of flow each of the first venous capillary receives from the first arterial order capillary. We plot the

depth of all pairs of first-order capillaries and color-code the fraction of each first venous order capillary receives (Figure 4.33A).

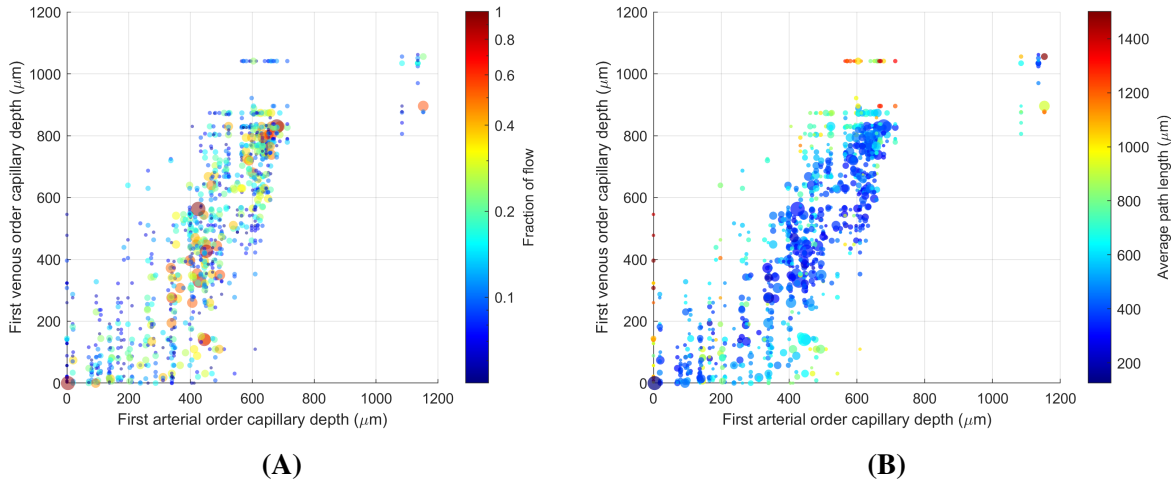


Figure 4.33. Scatter plot of the depth of the first order arterial capillaries and their draining first order venous capillaries. (A) The size and color of the dots show the fraction of flow from the arterial end capillaries to the venous end capillaries. (B) The size and color of the dots show the average capillary path length from the arterial end capillaries to the venous end capillaries

As expected, the scatter falls along the diagonal line, suggesting the flows exit the capillary bed at the same cortical depth as it enters. In addition, there seem to be a few first venous capillaries between 400 to 600 μm deep that mainly drain the blood from their corresponding first arterial order capillary. Regarding the capillary path length from the artery side to the vine size (Figure 4.33B), the average path length is about 400 μm and the capillary bed at depth 400 to 600 μm seems to have shorter average path length than in the superficial layer. Given the higher vessel length density in layer IV, further examining the density of the arterial/venous branch density in different cortical layers is required.

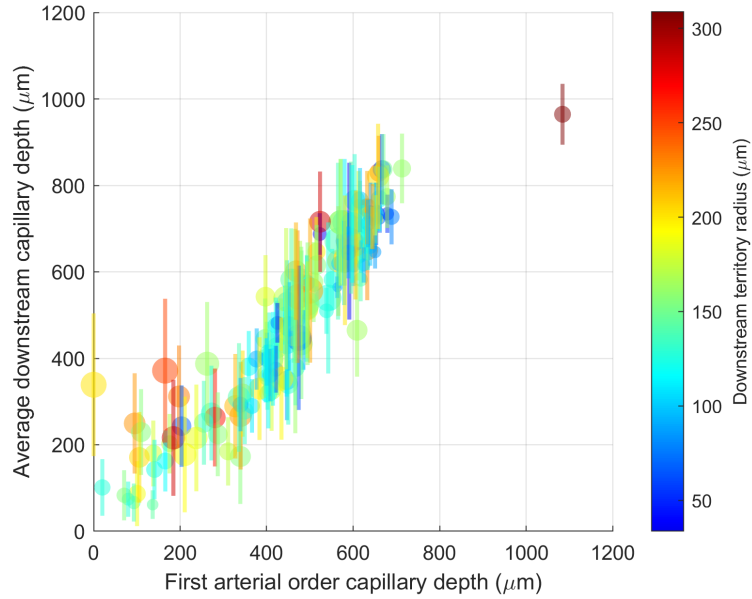


Figure 4.34. Scatter plot of the depth of the first-order arterial capillaries and the average depth of their downstream capillaries. Bars show the standard deviation of the downstream capillary depth. The dot color shows the effective radius of the downstream capillary tissue territory defined by distance transform.

Finally, we examine the perfusion territory of the first arterial order capillaries. We use Q_F to compute the mean and variance of the downstream capillary cortical depth and use Q_B to compute the weighted total downstream capillary nearest tissue volume, which is further converted to the effective tissue radius that corresponds to a sphere of the same volume. Again, the downstream capillaries lie on a similar cortical depth as the arterial side entrance, with a standard deviation of about $100 \mu m$ (Figure 4.34). The effective tissue radius is around 100 to $150 \mu m$.

4.8.5 Discussion

Previous studies used Monte Carlo simulation to study the effect of local blood flow regulation and estimate red blood cell transient time. The computational cost of these numerical studies prohibits systematic analysis of the parameter space. Under our assumptions, we derived analytical expressions for the effect of selective vessel radius modulation and the average

trajectory statistics. These results provide guidance for systematic analysis of the effect of vasodilation or occlusion in the local network. The average trajectory statistics summarize the results of Monte Carlo simulations that requires a large number of samples due to the complexity of the network.

The formula derived above dramatically reduces the computational cost for analyzing the micro-circulation using large-scale anatomically accurate network reconstruction. The limitations of our approach are mainly from the assumption. As Monte Carlo simulation samples the trajectories of many red blood cells, the estimate of the statistical quantities can be computed straightforwardly. Derivation for the variance of the trajectory statistics is one of our future directions. Our result on perturbation analysis is simply a linear response result. Loosing the assumption to include the biphasic nature of the blood flow requires more advanced analysis. However, once the biphasic flow is solved, the average trajectory statistics can again be applied directly.

4.8.6 Conclusion

We apply analytical and numerical approaches to study the cerebral blood flow regulation in the linear-response regime. The analytical results obtained from the linear model disentangle factors involved in linear responses in flow patterns due to single vessel dilation, constriction, or occlusion. We apply our method to study the blood flow pattern in a $(1.072mm)^3$ volume of mouse cortical vascular network. Our results are consistent with various prior studies obtained by Monte Carlo simulation that require much more computation resources. We will continue to study the distributions and statistics of the flow trajectory in the network analytically and apply these analytical results to study the blood flow pattern in different brain regions later when the data becomes available.

4.9 Acknowledgment

Chapter 2 to 4, in part, is a reprint of the material as it appears in X. Ji, T. Ferreira, B. Friedman, Rui Liu, H. Liechty, E. Bas, J. Chandrashekar, D. Kleinfeld, Brain microvasculature has a common topology with local differences in geometry that match metabolic load, *Neuron*, Volume 109, Issue 7, 2021, Pages 1168-1187.e13. The dissertation author was the primary investigator and first author of this paper.

4.10 Supplemental material

4.10.1 Quantified network features

Link features

Vessel segment i with n_i skeleton voxels was recorded as an ordered set:

$$E_i = \{\mathbf{x}_1^{(i)}, \mathbf{x}_2^{(i)}, \dots, \mathbf{x}_{n_i}^{(i)}\}, \quad (4.51)$$

where voxel at $\mathbf{x}_j^{(i)}$ was the 26-neighbor of voxels at $\mathbf{x}_{j-1}^{(i)}$ and $\mathbf{x}_{j+1}^{(i)}$. Each skeleton voxel j was associated with a radius and the radius of the vessel segment r_i was defined as the median of its skeleton voxel radii.

- Length: $l_i = \sum_{j=1}^{n_i-1} \|\mathbf{x}_j^{(i)} - \mathbf{x}_{j+1}^{(i)}\|_2$
- End-to-end vector: $\mathbf{V}_e^{(i)} = \mathbf{x}_{n_i}^{(i)} - \mathbf{x}_1^{(i)}$
- End-to-end distance: $d_{ep}^{(i)} = \|\mathbf{V}_e^{(i)}\|_2$
- Orientation vector: $\mathbf{v}_e^{(i)} = \mathbf{V}_e^{(i)} / d_{ep}^{(i)}$
- Surface area: $S_i = 2\pi r_i l_i$
- Volume: $V_i = \pi r_i^2 l_i$

- Straightness: $s_i = d_{ep}^{(i)} / l_i$
- Tangent vector at endpoint $\mathbf{x}_1^{(i)}$ ($\mathbf{x}_{n_i}^{(i)}$): $\mathbf{v}_{t,1}^{(i)}$ ($\mathbf{v}_{t,2}^{(i)}$) was the leading principle component of the 3D coordinate of the first (last) 10 (or n_i) skeleton voxels in the vessel segment i , with negative (positive) inner product with the orientation vector $\mathbf{v}_e^{(i)}$ ($\mathbf{v}_e^{(i)} \cdot \mathbf{v}_{t,1}^{(i)} < 0$, $\mathbf{v}_e^{(i)} \cdot \mathbf{v}_{t,2}^{(i)} > 0$).
- Is capillary: Q_i equals 1 if the link has radius r_i no greater than r_{cap} and 0 otherwise.
- Capillary branch order: geodesic distance to the nearest non-capillary
- Number of edges in the shortest loop: number of vessel segments in the shortest loop measured by Euclidean distance
- Average, minimum, maximum, standard deviation, coefficient of variance of vessel segment radius, and the radius at the vessel segment endpoints.
- Center of mass of vessel segment, and three principle components of the segment skeleton coordinates
- The Euclidean path length and geodesic distance from capillary segments and their nearest non-capillary in the 1072-cube
- Length of the shortest loop: sum of the Euclidean lengths of the vessel segments in the shortest loop
- Average distance between the segment skeleton voxels and the brain surface

Node features

The node connected components contained one or more skeleton voxels and were stored as unordered set of skeleton voxel coordinates:

$$N_i = \{\mathbf{x}_1^{(i)}, \mathbf{x}_2^{(i)}, \dots, \mathbf{x}_{n_i}^{(i)}\} \quad (4.52)$$

- Branch to the nearest node: number of vessel segments in the shortest path from node i to its nearest node
- Maximum, median, and minimum branch angles between link tangent vectors: $\phi_{t,max}$, $\phi_{t,med}$, $\phi_{t,min}$ were the largest, median and smallest angle (in degree) between the tangent vectors of the vessel segments (link) endpoints that connected to node N_i
- Maximum, median, and minimum branch angles between link end-to-end vectors: $\phi_{e,max}$, $\phi_{e,med}$, $\phi_{e,min}$ were the largest, median and smallest angle (in degree) between end-to-end vectors of the vessel segments (link) that connected to node N_i
- $|\hat{n}_{t12} \cdot \mathbf{v}_{t3}|$: The tangent vectors of the two connected endpoints were used to define the normal unit vector $\hat{n} = \frac{\mathbf{v}_{t1} \times \mathbf{v}_{t2}}{\|\mathbf{v}_{t1} \times \mathbf{v}_{t2}\|_2}$. The absolute value of the inner product between the normal unit vector $\hat{n}$ and the third tangent vector $\mathbf{v}_{t3}$ quantified the how close the connected vessel branches were on the same plane.
- Center of mass: $\mathbf{x}_{nc}^{(i)} = \frac{1}{n_i} \sum_{j=1}^{n_i} \mathbf{x}_j^{(i)}$
- Euclidean distance to the nearest node
- The Euclidean path length and geodesic distance to the neighboring nodes
- Node radius: average distance transform value of the node voxels
- Radial node density function: for each node, bin its neighboring nodes by the Euclidean distance between the center of mass, and normalized by the volume of the concentric spherical layer
- Connected neighbor distance ranking: Euclidean distance ranking of the connected node neighbors among all the neighboring nodes
- Minimum, median and maximum length of the connected vessel segments
- Average distance between the node center of mass and the brain surface

240-cube features

Vessel segments were extended objects that might be partially present inside the 240-cube.

For a 240-cube with n_E vessel segments passing by:

- Vessel length density: $\rho_l = \frac{1}{V_c} \sum_i^{n_E} l_i f_i$, where f_i was the fraction of vessel skeleton voxels inside the bounding box of the 240-cube and V_c was the volume of the 240-cube ($V_c = 240^3 \mu m^3$)
- Capillary length density: $\rho_{lc} = \frac{1}{V_c} \sum_i^{n_E} l_i f_i Q_i$
- Similarly, the vessel (capillary) surface area density and volume density could be defined

Distance transform with respect to the reconstructed vessel mask gave the distance transform field $d(\mathbf{x})$ whose value at $\mathbf{x}$ was the minimum distance between the tissue at $\mathbf{x}$ and the nearest reconstructed vessel wall. The same reconstruction was used for computing the normalized pO_2 field $u(\mathbf{x})$, see section Diffusive substrate transportation and consumption. Local extrema of $d(\mathbf{x})$ and $u(\mathbf{x})$ were found by a $50 \mu m$ width moving maximum /minimum filter.

- Average vessel-tissue distance: $\bar{d} = \frac{\int_{V_c} d(\mathbf{x}) d\mathbf{x}}{V_c}$
- d_{lm} : average value of the DT local maxima inside the cube ($\overline{d_{max}}$ in the main text)
- u_{lm} : average value of the pO_2 local minima inside the cube ($\overline{c_{min}}$ in the main text)
- d_{ulm} : average distance between normalized pO_2 local minima and their nearest vessels inside the cube ($\overline{d_{cmin}}$ in the main text)
- u_{dlm} : average value of the normalized pO_2 value at the local DT maxima inside the cube

4.10.2 Tables

Table 4.2. Regional vessel length densities. Computed from the densities in 240-cubes in the region. Small regions without enough 240-cubes are shown as “-”.

Structure name	Acronym	This study					
		Vessel length density (m/mm ³)			Capillary length density (m/mm ³)		
		Mean	SE	SD	Mean	SE	SD
root	root	0.873	0.022	0.243	0.833	0.021	0.237
Basic cell groups and regions	grey	0.896	0.017	0.224	0.860	0.016	0.220
Cerebrum	CH	0.831	0.014	0.194	0.800	0.017	0.191
Cerebral cortex	CTX	0.845	0.015	0.205	0.815	0.018	0.201
Cortical plate	CTXpl	0.858	0.015	0.204	0.828	0.018	0.200
Isocortex	Isocortex	0.911	0.017	0.161	0.883	0.019	0.158
Frontal pole cerebral cortex	FRP	0.896	0.053	0.044	0.869	0.057	0.045
Frontal pole layer 1	FRP1	-	-	-	-	-	-
Frontal pole layer 2/3	FRP2/3	-	-	-	-	-	-
Frontal pole layer 5	FRP5	0.902	0.071	0.010	0.871	0.077	0.012
Somatomotor areas	MO	0.934	0.030	0.113	0.905	0.031	0.112
Primary motor area	MOp	0.952	0.037	0.127	0.923	0.038	0.125
Primary motor area Layer 1	MOp1	-	-	-	-	-	-
Primary motor area Layer 2/3	MOp2/3	1.007	0.026	0.093	0.974	0.030	0.093
Primary motor area Layer 5	MOp5	0.998	0.030	0.085	0.968	0.030	0.085
Primary motor area Layer 6a	MOp6a	0.810	0.067	0.082	0.786	0.068	0.083
Primary motor area Layer 6b	MOp6b	-	-	-	-	-	-
Secondary motor area	MOs	0.919	0.023	0.089	0.889	0.025	0.089
Secondary motor area layer 1	MOs1	-	-	-	-	-	-
Secondary motor area layer 2/3	MOs2/3	0.949	0.022	0.071	0.915	0.022	0.073
Secondary motor area layer 5	MOs5	0.938	0.019	0.072	0.909	0.021	0.072
Secondary motor area layer 6a	MOs6a	0.827	0.062	0.073	0.797	0.062	0.074
Secondary motor area layer 6b	MOs6b	-	-	-	-	-	-
Somatosensory areas	SS	1.011	0.022	0.163	0.981	0.021	0.159
Primary somatosensory area	SSp	1.018	0.028	0.169	0.988	0.028	0.164
Primary somatosensory area nose	SSp-n	1.080	0.028	0.152	1.046	0.025	0.149
Primary somatosensory area nose layer 1	SSp-n1	-	-	-	-	-	-
Primary somatosensory area nose layer 2/3	SSp-n2/3	1.065	0.035	0.046	1.028	0.031	0.052
Primary somatosensory area nose layer 4	SSp-n4	1.253	0.055	0.059	1.219	0.049	0.054
Primary somatosensory area nose layer 5	SSp-n5	1.102	0.058	0.088	1.067	0.053	0.086
Primary somatosensory area nose layer 6a	SSp-n6a	0.892	0.044	0.107	0.864	0.041	0.104
Primary somatosensory area nose layer 6b	SSp-n6b	-	-	-	-	-	-
Primary somatosensory area barrel field	SSp-bfd	1.000	0.034	0.165	0.971	0.032	0.160
Primary somatosensory area barrel field layer 1	SSp-bfd1	-	-	-	-	-	-
Primary somatosensory area barrel field layer 2/3	SSp-bfd2/3	1.027	0.050	0.092	0.991	0.049	0.090
Primary somatosensory area barrel field layer 4	SSp-bfd4	1.168	0.054	0.101	1.133	0.049	0.099
Primary somatosensory area barrel field layer 5	SSp-bfd5	1.020	0.036	0.089	0.988	0.038	0.086
Primary somatosensory area barrel field layer 6a	SSp-bfd6a	0.794	0.071	0.101	0.775	0.071	0.100
Primary somatosensory area barrel field layer 6b	SSp-bfd6b	-	-	-	-	-	-
Primary somatosensory area lower limb	SSp-l	0.960	0.046	0.155	0.931	0.048	0.153
Primary somatosensory area lower limb layer 1	SSp-l1	-	-	-	-	-	-
Primary somatosensory area lower limb layer 2/3	SSp-l2/3	0.990	0.031	0.061	0.956	0.031	0.067
Primary somatosensory area lower limb layer 4	SSp-l4	-	-	-	-	-	-
Primary somatosensory area lower limb layer 5	SSp-l5	1.037	0.043	0.065	1.005	0.042	0.063
Primary somatosensory area lower limb layer 6a	SSp-l6a	0.714	0.119	0.081	0.695	0.125	0.080
Primary somatosensory area lower limb layer 6b	SSp-l6b	-	-	-	-	-	-
Primary somatosensory area mouth	SSp-m	1.063	0.026	0.165	1.031	0.023	0.162
Primary somatosensory area mouth layer 1	SSp-m1	-	-	-	-	-	-
Primary somatosensory area mouth layer 2/3	SSp-m2/3	1.108	0.051	0.095	1.073	0.053	0.096
Primary somatosensory area mouth layer 4	SSp-m4	-	-	-	-	-	-
Primary somatosensory area mouth layer 5	SSp-m5	1.098	0.024	0.077	1.065	0.022	0.073
Primary somatosensory area mouth layer 6a	SSp-m6a	0.859	0.038	0.096	0.833	0.035	0.095
Primary somatosensory area mouth layer 6b	SSp-m6b	-	-	-	-	-	-

Table 4.2 Regional vessel length densities. Computed from the densities in 240-cubes in the region. Small regions without enough 240-cubes are shown as “-”. (Continue)

Primary somatosensory area upper limb	SSp-ul	1.015	0.038	0.165	0.983	0.038	0.161
Primary somatosensory area upper limb layer 1	SSp-ul1	-	-	-	-	-	-
Primary somatosensory area upper limb layer 2/3	SSp-ul2/3	1.046	0.013	0.079	1.007	0.012	0.082
Primary somatosensory area upper limb layer 4	SSp-ul4	-	-	-	-	-	-
Primary somatosensory area upper limb layer 5	SSp-ul5	1.060	0.035	0.065	1.028	0.029	0.061
Primary somatosensory area upper limb layer 6a	SSp-ul6a	0.783	0.066	0.078	0.761	0.069	0.078
Primary somatosensory area upper limb layer 6b	SSp-ul6b	-	-	-	-	-	-
Primary somatosensory area trunk	SSp-tr	0.945	0.047	0.132	0.919	0.047	0.129
Primary somatosensory area trunk layer 1	SSp-tr1	-	-	-	-	-	-
Primary somatosensory area trunk layer 2/3	SSp-tr2/3	0.948	0.028	0.050	0.910	0.042	0.049
Primary somatosensory area trunk layer 4	SSp-tr4	-	-	-	-	-	-
Primary somatosensory area trunk layer 5	SSp-tr5	0.991	0.061	0.069	0.966	0.064	0.071
Primary somatosensory area trunk layer 6a	SSp-tr6a	-	-	-	-	-	-
Primary somatosensory area trunk layer 6b	SSp-tr6b	-	-	-	-	-	-
Primary somatosensory area unassigned	SSp-un	1.056	0.026	0.121	1.026	0.023	0.117
Supplemental somatosensory area	SSs	0.994	0.013	0.137	0.962	0.013	0.135
Supplemental somatosensory area layer 1	SSs1	-	-	-	-	-	-
Supplemental somatosensory area layer 2/3	SSs2/3	0.948	0.046	0.122	0.911	0.045	0.121
Supplemental somatosensory area layer 4	SSs4	-	-	-	-	-	-
Supplemental somatosensory area layer 5	SSs5	1.020	0.019	0.082	0.991	0.017	0.081
Supplemental somatosensory area layer 6a	SSs6a	0.864	0.024	0.067	0.840	0.022	0.069
Supplemental somatosensory area layer 6b	SSs6b	-	-	-	-	-	-
Gustatory areas	GU	0.817	0.065	0.106	0.790	0.066	0.105
Gustatory areas layer 1	GU1	-	-	-	-	-	-
Gustatory areas layer 2/3	GU2/3	-	-	-	-	-	-
Gustatory areas layer 4	GU4	-	-	-	-	-	-
Gustatory areas layer 5	GU5	0.818	0.074	0.077	0.792	0.072	0.081
Gustatory areas layer 6a	GU6a	0.733	0.031	0.054	0.706	0.032	0.059
Gustatory areas layer 6b	GU6b	-	-	-	-	-	-
Visceral area	VISC	0.814	0.025	0.095	0.786	0.025	0.099
Visceral area layer 1	VISC1	-	-	-	-	-	-
Visceral area layer 2/3	VISC2/3	-	-	-	-	-	-
Visceral area layer 4	VISC4	-	-	-	-	-	-
Visceral area layer 5	VISC5	0.825	0.027	0.051	0.798	0.024	0.055
Visceral area layer 6a	VISC6a	0.698	0.062	0.041	0.673	0.063	0.042
Visceral area layer 6b	VISC6b	-	-	-	-	-	-
Auditory areas	AUD	0.946	0.014	0.137	0.917	0.013	0.134
Dorsal auditory area	AUDd	0.966	0.040	0.108	0.931	0.039	0.106
Dorsal auditory area layer 1	AUDd1	-	-	-	-	-	-
Dorsal auditory area layer 2/3	AUDd2/3	0.928	0.085	0.056	0.896	0.079	0.047
Dorsal auditory area layer 4	AUDd4	-	-	-	-	-	-
Dorsal auditory area layer 5	AUDd5	0.963	0.052	0.052	0.916	0.056	0.056
Dorsal auditory area layer 6a	AUDd6a	-	-	-	-	-	-
Dorsal auditory area layer 6b	AUDd6b	-	-	-	-	-	-
Primary auditory area	AUDp	0.993	0.021	0.141	0.965	0.020	0.138
Primary auditory area layer 1	AUDp1	-	-	-	-	-	-
Primary auditory area layer 2/3	AUDp2/3	-	-	-	-	-	-
Primary auditory area layer 4	AUDp4	-	-	-	-	-	-
Primary auditory area layer 5	AUDp5	0.992	0.014	0.085	0.968	0.012	0.082
Primary auditory area layer 6a	AUDp6a	-	-	-	-	-	-
Primary auditory area layer 6b	AUDp6b	-	-	-	-	-	-
Posterior auditory area	AUDpo	0.916	0.042	0.082	0.892	0.043	0.082
Posterior auditory area layer 1	AUDpo1	-	-	-	-	-	-
Posterior auditory area layer 2/3	AUDpo2/3	-	-	-	-	-	-
Posterior auditory area layer 4	AUDpo4	-	-	-	-	-	-
Posterior auditory area layer 5	AUDpo5	0.922	0.036	0.004	0.901	0.034	0.004
Posterior auditory area layer 6a	AUDpo6a	-	-	-	-	-	-
Posterior auditory area layer 6b	AUDpo6b	-	-	-	-	-	-
Ventral auditory area	AUDv	0.868	0.028	0.103	0.841	0.028	0.102
Ventral auditory area layer 1	AUDv1	-	-	-	-	-	-
Ventral auditory area layer 2/3	AUDv2/3	-	-	-	-	-	-
Ventral auditory area layer 4	AUDv4	-	-	-	-	-	-
Ventral auditory area layer 5	AUDv5	0.892	0.029	0.070	0.864	0.029	0.068

Table 4.2 Regional vessel length densities. Computed from the densities in 240-cubes in the region. Small regions without enough 240-cubes are shown as “-“. (Continue)

Ventral auditory area layer 6a	AUDv6a	-	-	-	-	-	-
Ventral auditory area layer 6b	AUDv6b	-	-	-	-	-	-
Visual areas	VIS	0.846	0.016	0.109	0.821	0.018	0.106
Anterolateral visual area	VISal	0.848	0.020	0.087	0.824	0.019	0.084
Anterolateral visual area layer 1	VISal1	-	-	-	-	-	-
Anterolateral visual area layer 2/3	VISal2/3	-	-	-	-	-	-
Anterolateral visual area layer 4	VISal4	-	-	-	-	-	-
Anterolateral visual area layer 5	VISal5	-	-	-	-	-	-
Anterolateral visual area layer 6a	VISal6a	-	-	-	-	-	-
Anterolateral visual area layer 6b	VISal6b	-	-	-	-	-	-
Anteromedial visual area	VISam	0.832	0.052	0.086	0.804	0.053	0.085
Anteromedial visual area layer 1	VISam1	-	-	-	-	-	-
Anteromedial visual area layer 2/3	VISam2/3	-	-	-	-	-	-
Anteromedial visual area layer 4	VISam4	-	-	-	-	-	-
Anteromedial visual area layer 5	VISam5	-	-	-	-	-	-
Anteromedial visual area layer 6a	VISam6a	-	-	-	-	-	-
Anteromedial visual area layer 6b	VISam6b	-	-	-	-	-	-
Lateral visual area	VISI	0.826	0.018	0.085	0.803	0.015	0.083
Lateral visual area layer 1	VISI1	-	-	-	-	-	-
Lateral visual area layer 2/3	VISI2/3	-	-	-	-	-	-
Lateral visual area layer 4	VISI4	-	-	-	-	-	-
Lateral visual area layer 5	VISI5	0.814	0.041	0.001	0.797	0.047	0.004
Lateral visual area layer 6a	VISI6a	-	-	-	-	-	-
Lateral visual area layer 6b	VISI6b	-	-	-	-	-	-
Primary visual area	VISp	0.873	0.023	0.109	0.847	0.025	0.106
Primary visual area layer 1	VISp1	-	-	-	-	-	-
Primary visual area layer 2/3	VISp2/3	0.901	0.017	0.054	0.869	0.018	0.060
Primary visual area layer 4	VISp4	-	-	-	-	-	-
Primary visual area layer 5	VISp5	0.850	0.027	0.035	0.825	0.032	0.036
Primary visual area layer 6a	VISp6a	0.706	0.046	0.000	0.695	0.035	0.001
Primary visual area layer 6b	VISp6b	-	-	-	-	-	-
Posterolateral visual area	VISpl	0.824	0.030	0.105	0.800	0.030	0.099
Posterolateral visual area layer 1	VISpl1	-	-	-	-	-	-
Posterolateral visual area layer 2/3	VISpl2/3	-	-	-	-	-	-
Posterolateral visual area layer 4	VISpl4	-	-	-	-	-	-
Posterolateral visual area layer 5	VISpl5	-	-	-	-	-	-
Posterolateral visual area layer 6a	VISpl6a	-	-	-	-	-	-
Posterolateral visual area layer 6b	VISpl6b	-	-	-	-	-	-
posteromedial visual area	VISpm	0.836	0.030	0.082	0.810	0.030	0.082
posteromedial visual area layer 1	VISpm1	-	-	-	-	-	-
posteromedial visual area layer 2/3	VISpm2/3	-	-	-	-	-	-
posteromedial visual area layer 4	VISpm4	-	-	-	-	-	-
posteromedial visual area layer 5	VISpm5	0.869	0.090	0.016	0.843	0.086	0.017
posteromedial visual area layer 6a	VISpm6a	-	-	-	-	-	-
posteromedial visual area layer 6b	VISpm6b	-	-	-	-	-	-
Laterointermediate area	VISli	0.833	0.028	0.080	0.807	0.022	0.083
Postrhinal area	VISpor	0.775	0.042	0.084	0.753	0.038	0.084
Postrhinal area layer 5	VISpor5	0.783	0.042	0.048	0.764	0.038	0.044
Anterior cingulate area	ACA	0.893	0.025	0.105	0.870	0.027	0.104
Anterior cingulate area dorsal part	ACAd	0.898	0.018	0.086	0.875	0.021	0.086
Anterior cingulate area dorsal part layer 1	ACAd1	-	-	-	-	-	-
Anterior cingulate area dorsal part layer 2/3	ACAd2/3	-	-	-	-	-	-
Anterior cingulate area dorsal part layer 5	ACAd5	0.910	0.024	0.065	0.887	0.027	0.063
Anterior cingulate area dorsal part layer 6a	ACAd6a	-	-	-	-	-	-
Anterior cingulate area dorsal part layer 6b	ACAd6b	-	-	-	-	-	-
Anterior cingulate area ventral part	ACAv	0.901	0.045	0.112	0.878	0.046	0.111
Anterior cingulate area ventral part layer 1	ACAv1	-	-	-	-	-	-
Anterior cingulate area ventral part layer 2/3	ACAv2/3	-	-	-	-	-	-
Anterior cingulate area ventral part layer 5	ACAv5	0.895	0.019	0.084	0.875	0.019	0.085
Anterior cingulate area ventral part 6a	ACAv6a	-	-	-	-	-	-
Anterior cingulate area ventral part 6b	ACAv6b	-	-	-	-	-	-
Prelimbic area	PL	0.773	0.025	0.064	0.750	0.026	0.066
Prelimbic area layer 1	PL1	-	-	-	-	-	-
Prelimbic area layer 2/3	PL2/3	-	-	-	-	-	-
Prelimbic area layer 5	PL5	0.783	0.023	0.052	0.764	0.022	0.056
Prelimbic area layer 6a	PL6a	0.701	0.045	0.046	0.676	0.044	0.048
Prelimbic area layer 6b	PL6b	-	-	-	-	-	-
Infralimbic area	ILA	0.720	0.050	0.040	0.699	0.050	0.041
Infralimbic area layer 1	ILA1	-	-	-	-	-	-

Table 4.2 Regional vessel length densities. Computed from the densities in 240-cubes in the region. Small regions without enough 240-cubes are shown as “-”. (Continue)

Infralimbic area layer 2/3	ILA2/3	-	-	-	-	-	-
Infralimbic area layer 5	ILA5	0.725	0.055	0.039	0.705	0.054	0.041
Infralimbic area layer 6a	ILA6a	-	-	-	-	-	-
Infralimbic area layer 6b	ILA6b	-	-	-	-	-	-
Orbital area	ORB	0.882	0.012	0.133	0.849	0.016	0.133
Orbital area lateral part	ORBI	0.955	0.013	0.122	0.923	0.011	0.124
Orbital area lateral part layer 1	ORBI1	-	-	-	-	-	-
Orbital area lateral part layer 2/3	ORBI2/3	-	-	-	-	-	-
Orbital area lateral part layer 5	ORBI5	0.965	0.032	0.083	0.935	0.031	0.089
Orbital area lateral part layer 6a	ORBI6a	0.761	0.039	0.041	0.727	0.039	0.039
Orbital area lateral part layer 6b	ORBI6b	-	-	-	-	-	-
Orbital area medial part	ORBm	0.768	0.009	0.079	0.735	0.022	0.080
Orbital area medial part layer 1	ORBm1	-	-	-	-	-	-
Orbital area medial part layer 2/3	ORBm2/3	-	-	-	-	-	-
Orbital area medial part layer 5	ORBm5	0.771	0.025	0.051	0.749	0.022	0.047
Orbital area medial part layer 6a	ORBm6a	-	-	-	-	-	-
Orbital area ventrolateral part	ORBvl	0.866	0.020	0.093	0.835	0.022	0.093
Orbital area ventrolateral part layer 1	ORBvl1	-	-	-	-	-	-
Orbital area ventrolateral part layer 2/3	ORBvl2/3	-	-	-	-	-	-
Orbital area ventrolateral part layer 5	ORBvl5	0.885	0.031	0.062	0.860	0.030	0.062
Orbital area ventrolateral part layer 6a	ORBvl6a	-	-	-	-	-	-
Orbital area ventrolateral part layer 6b	ORBvl6b	-	-	-	-	-	-
Agranular insular area	AI	0.758	0.019	0.104	0.733	0.020	0.105
Agranular insular area dorsal part	AId	0.793	0.019	0.088	0.769	0.021	0.088
Agranular insular area dorsal part layer 1	AId1	-	-	-	-	-	-
Agranular insular area dorsal part layer 2/3	AId2/3	-	-	-	-	-	-
Agranular insular area dorsal part layer 5	AId5	0.790	0.028	0.072	0.768	0.030	0.072
Agranular insular area dorsal part layer 6a	AId6a	0.751	0.019	0.052	0.729	0.019	0.055
Agranular insular area dorsal part layer 6b	AId6b	-	-	-	-	-	-
Agranular insular area posterior part	Alp	0.667	0.028	0.058	0.643	0.030	0.059
Agranular insular area posterior part layer 1	Alp1	-	-	-	-	-	-
Agranular insular area posterior part layer 2/3	Alp2/3	0.656	0.039	0.045	0.629	0.041	0.046
Agranular insular area posterior part layer 5	Alp5	0.707	0.036	0.044	0.684	0.037	0.043
Agranular insular area posterior part layer 6a	Alp6a	-	-	-	-	-	-
Agranular insular area posterior part layer 6b	Alp6b	-	-	-	-	-	-
Agranular insular area ventral part	Alv	0.802	0.025	0.104	0.770	0.025	0.102
Agranular insular area ventral part layer 1	Alv1	-	-	-	-	-	-
Agranular insular area ventral part layer 2/3	Alv2/3	-	-	-	-	-	-
Agranular insular area ventral part layer 5	Alv5	0.813	0.024	0.080	0.785	0.023	0.087
Agranular insular area ventral part layer 6a	Alv6a	-	-	-	-	-	-
Agranular insular area ventral part layer 6b	Alv6b	-	-	-	-	-	-
Retrosplenial area	RSP	0.966	0.042	0.152	0.936	0.044	0.149
Retrosplenial area lateral agranular part	RSPagl	0.870	0.030	0.084	0.842	0.034	0.085
Retrosplenial area lateral agranular part layer 1	RSPagl1	-	-	-	-	-	-
Retrosplenial area lateral agranular part layer 2/3	RSPagl2/3	-	-	-	-	-	-
Retrosplenial area lateral agranular part layer 5	RSPagl5	0.920	0.036	0.080	0.893	0.034	0.078
Retrosplenial area lateral agranular part layer 6a	RSPagl6a	-	-	-	-	-	-
Retrosplenial area lateral agranular part layer 6b	RSPagl6b	-	-	-	-	-	-
Retrosplenial area dorsal part	RSPd	0.987	0.040	0.123	0.961	0.042	0.122
Retrosplenial area dorsal part layer 1	RSPd1	-	-	-	-	-	-
Retrosplenial area dorsal part layer 2/3	RSPd2/3	-	-	-	-	-	-
Retrosplenial area dorsal part layer 5	RSPd5	1.060	0.045	0.067	1.031	0.047	0.068
Retrosplenial area dorsal part layer 6a	RSPd6a	0.808	0.067	0.077	0.785	0.068	0.076
Retrosplenial area dorsal part layer 6b	RSPd6b	-	-	-	-	-	-
Retrosplenial area ventral part	RSPv	1.037	0.062	0.138	0.999	0.059	0.140
Retrosplenial area ventral part layer 1	RSPv1	-	-	-	-	-	-
Retrosplenial area ventral part layer 2/3	RSPv2/3	-	-	-	-	-	-
Retrosplenial area ventral part layer 5	RSPv5	1.076	0.031	0.073	1.049	0.033	0.074
Retrosplenial area ventral part layer 6a	RSPv6a	0.860	0.215	0.059	0.843	0.211	0.060
Retrosplenial area ventral part layer 6b	RSPv6b	-	-	-	-	-	-
Posterior parietal association areas	PTLp	0.870	0.041	0.121	0.844	0.042	0.119

Table 4.2 Regional vessel length densities. Computed from the densities in 240-cubes in the region. Small regions without enough 240-cubes are shown as “-”. (Continue)

Anterior area	VISa	0.870	0.047	0.104	0.844	0.049	0.103
Anterior area layer 2/3	VISa2/3	0.874	0.055	0.075	0.844	0.053	0.076
Rostrolateral visual area	VISrl	0.884	0.032	0.121	0.858	0.032	0.116
Rostrolateral area layer 5	VISrl5	0.856	0.044	0.028	0.836	0.047	0.032
Rostrolateral area layer 6b	VISrl6b	-	-	-	-	-	-
Temporal association areas	TEa	0.728	0.026	0.087	0.704	0.030	0.089
Temporal association areas layer 1	TEa1	-	-	-	-	-	-
Temporal association areas layer 2/3	TEa2/3	-	-	-	-	-	-
Temporal association areas layer 4	TEa4	-	-	-	-	-	-
Temporal association areas layer 5	TEa5	0.763	0.028	0.062	0.739	0.031	0.060
Temporal association areas layer 6a	TEa6a	-	-	-	-	-	-
Temporal association areas layer 6b	TEa6b	-	-	-	-	-	-
Perirhinal area	PERl	-	-	-	-	-	-
Perirhinal area layer 1	PERl1	-	-	-	-	-	-
Perirhinal area layer 2/3	PERl2/3	-	-	-	-	-	-
Perirhinal area layer 5	PERl5	-	-	-	-	-	-
Perirhinal area layer 6a	PERl6a	-	-	-	-	-	-
Perirhinal area layer 6b	PERl6b	-	-	-	-	-	-
Ectorhinal area	ECT	0.677	0.028	0.074	0.655	0.027	0.078
Ectorhinal area/Layer 1	ECT1	-	-	-	-	-	-
Ectorhinal area/Layer 2/3	ECT2/3	-	-	-	-	-	-
Ectorhinal area/Layer 5	ECT5	0.737	0.025	0.033	0.717	0.025	0.033
Ectorhinal area/Layer 6a	ECT6a	-	-	-	-	-	-
Ectorhinal area/Layer 6b	ECT6b	-	-	-	-	-	-
Olfactory areas	OLF	0.809	0.067	0.294	0.782	0.070	0.284
Main olfactory bulb	MOB	0.887	0.117	0.346	0.861	0.120	0.335
Accessory olfactory bulb	AOB	0.738	0.078	0.087	0.692	0.061	0.076
Accessory olfactory bulb glomerular layer	AOBgl	-	-	-	-	-	-
Accessory olfactory bulb granular layer	AOBgr	-	-	-	-	-	-
Accessory olfactory bulb mitral layer	AOBmi	0.698	0.060	0.022	0.686	0.054	0.023
Anterior olfactory nucleus	AON	0.780	0.026	0.157	0.748	0.026	0.147
Taenia tecta	TT	0.766	0.047	0.121	0.727	0.029	0.103
Taenia tecta dorsal part	TTd	0.776	0.101	0.098	0.730	0.075	0.076
Taenia tecta ventral part	TTv	0.740	0.040	0.100	0.712	0.052	0.096
Dorsal peduncular area	DP	0.784	0.060	0.086	0.752	0.049	0.076
Piriform area	PIR	0.738	0.010	0.166	0.718	0.010	0.162
Nucleus of the lateral olfactory tract	NLOT	0.858	0.050	0.061	0.836	0.056	0.063
Nucleus of the lateral olfactory tract molecular layer	NLOT1	-	-	-	-	-	-
Nucleus of the lateral olfactory tract layer 3	NLOT3	-	-	-	-	-	-
Cortical amygdalar area	COA	0.582	0.014	0.084	0.564	0.014	0.085
Cortical amygdalar area anterior part	COAa	0.678	0.023	0.059	0.661	0.025	0.061
Cortical amygdalar area posterior part	COAp	0.554	0.017	0.065	0.535	0.017	0.068
Cortical amygdalar area posterior part lateral zone	COApl	0.559	0.024	0.061	0.541	0.028	0.061
Cortical amygdalar area posterior part medial zone	COApm	0.569	0.046	0.048	0.552	0.052	0.047
Piriform-amygdalar area	PAA	0.621	0.029	0.055	0.605	0.027	0.058
Postpiriform transition area	TR	0.592	0.015	0.064	0.570	0.011	0.063
Hippocampal formation	HPF	0.753	0.013	0.140	0.717	0.011	0.134
Hippocampal region	HIP	0.714	0.026	0.121	0.677	0.023	0.114
Ammon's horn	CA	0.684	0.024	0.120	0.652	0.020	0.113
Field CA1	CA1	0.658	0.026	0.121	0.628	0.024	0.112
Field CA1 pyramidal layer	CA1sp	-	-	-	-	-	-
Field CA2	CA2	-	-	-	-	-	-
Field CA3	CA3	0.714	0.033	0.098	0.684	0.026	0.098
Dentate gyrus	DG	0.748	0.039	0.090	0.708	0.035	0.091
Dentate gyrus molecular layer	DG-mo	0.810	0.094	0.048	0.743	0.085	0.073
Dentate gyrus polymorph layer	DG-po	-	-	-	-	-	-
Dentate gyrus granule cell layer	DG-sg	-	-	-	-	-	-
Fasciola cinerea	FC	-	-	-	-	-	-
Induseum griseum	IG	-	-	-	-	-	-
Retrohippocampal region	RHP	0.811	0.020	0.146	0.781	0.020	0.139
Entorhinal area	ENT	0.749	0.024	0.125	0.726	0.025	0.121
Entorhinal area lateral part	ENTl	0.689	0.025	0.091	0.668	0.023	0.091
Entorhinal area lateral part layer 5	ENTl5	0.653	0.024	0.061	0.631	0.027	0.061
Entorhinal area lateral part layer 6a	ENTl6a	0.654	0.038	0.057	0.636	0.037	0.061
Entorhinal area medial part dorsal zone	ENTm	0.835	0.026	0.117	0.808	0.028	0.110
Entorhinal area medial part ventral zone	ENTmv	-	-	-	-	-	-

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Parasubiculum	PAR	1.011	0.078	0.105	0.979	0.076	0.103
Postsubiculum	POST	1.018	0.036	0.099	0.964	0.025	0.099
Presubiculum	PRE	1.008	0.039	0.075	0.964	0.033	0.075
Subiculum	SUB	0.884	0.028	0.081	0.842	0.028	0.093
Prosubiculum	ProS	-	-	-	-	-	-
Cortical subplate	CTXsp	0.621	0.014	0.079	0.596	0.014	0.079
Clastrum	CLA	-	-	-	-	-	-
Endopiriform nucleus	EP	0.660	0.033	0.096	0.632	0.033	0.092
Endopiriform nucleus dorsal part	EPd	0.666	0.036	0.097	0.638	0.036	0.092
Endopiriform nucleus ventral part	EPv	-	-	-	-	-	-
Lateral amygdalar nucleus	LA	0.546	0.023	0.059	0.522	0.022	0.058
Basolateral amygdalar nucleus	BLA	0.641	0.010	0.070	0.614	0.009	0.070
Basolateral amygdalar nucleus anterior part	BLAa	0.692	0.019	0.053	0.665	0.020	0.056
Basolateral amygdalar nucleus posterior part	BLAp	0.603	0.023	0.051	0.580	0.021	0.053
Basolateral amygdalar nucleus ventral part	BLAv	0.590	0.029	0.046	0.569	0.029	0.040
Basomedial amygdalar nucleus	BMA	0.609	0.016	0.071	0.582	0.019	0.073
Basomedial amygdalar nucleus anterior part	BMAa	0.596	0.022	0.072	0.573	0.025	0.078
Basomedial amygdalar nucleus posterior part	BMAp	0.616	0.025	0.067	0.587	0.027	0.067
Posterior amygdalar nucleus	PA	0.686	0.054	0.076	0.665	0.052	0.086
Cerebral nuclei	CNU	0.791	0.027	0.139	0.759	0.026	0.140
Striatum	STR	0.799	0.028	0.136	0.768	0.026	0.136
Striatum dorsal region	STRd	0.849	0.031	0.106	0.817	0.027	0.108
Caudoputamen	CP	0.849	0.031	0.106	0.817	0.027	0.108
Striatum ventral region	STRv	0.809	0.010	0.112	0.782	0.011	0.111
Nucleus accumbens	ACB	0.817	0.014	0.114	0.788	0.013	0.114
Fundus of striatum	FS	-	-	-	-	-	-
Olfactory tubercle	OT	0.748	0.020	0.064	0.729	0.018	0.063
Lateral septal complex	LSX	0.627	0.055	0.101	0.596	0.054	0.102
Lateral septal nucleus	LS	0.633	0.052	0.102	0.605	0.051	0.101
Lateral septal nucleus caudal (caudodorsal) part	LSc	0.467	0.061	0.065	0.442	0.061	0.063
Lateral septal nucleus rostral (rostroventral) part	LSr	0.663	0.052	0.072	0.634	0.051	0.074
Lateral septal nucleus ventral part	LSv	0.684	0.031	0.055	0.653	0.032	0.059
Septofimbrial nucleus	SF	-	-	-	-	-	-
Septohippocampal nucleus	SH	-	-	-	-	-	-
Striatum-like amygdalar nuclei	sAMY	0.607	0.014	0.102	0.579	0.015	0.101
Anterior amygdalar area	AAA	0.818	0.072	0.096	0.776	0.067	0.100
Bed nucleus of the accessory olfactory tract	BA	-	-	-	-	-	-
Central amygdalar nucleus	CEA	0.603	0.013	0.081	0.573	0.013	0.080
Central amygdalar nucleus medial part	CEAm	0.615	0.013	0.078	0.586	0.015	0.080
Intercalated amygdalar nucleus	IA	-	-	-	-	-	-
Medial amygdalar nucleus	MEA	0.571	0.016	0.065	0.547	0.019	0.071
Pallidum	PAL	0.767	0.038	0.158	0.736	0.038	0.159
Pallidum dorsal region	PALd	0.843	0.069	0.128	0.814	0.062	0.130
Globus pallidus external segment	GPe	0.869	0.078	0.128	0.840	0.071	0.128
Globus pallidus internal segment	GPI	0.756	0.057	0.053	0.725	0.054	0.053
Pallidum ventral region	PALv	0.799	0.019	0.147	0.770	0.019	0.147
Substantia innominata	SI	0.747	0.022	0.093	0.720	0.020	0.095
Magnocellular nucleus	MA	1.098	0.046	0.088	1.065	0.054	0.081
Pallidum medial region	PALm	0.871	0.074	0.133	0.836	0.080	0.139
Medial septal complex	MSC	0.901	0.068	0.105	0.867	0.076	0.109
Medial septal nucleus	MS	-	-	-	-	-	-
Diagonal band nucleus	NDB	0.899	0.065	0.110	0.864	0.075	0.114
Triangular nucleus of septum	TRS	0.678	0.091	0.020	0.630	0.080	0.019
Pallidum caudal region	PALc	0.566	0.035	0.058	0.536	0.033	0.063
Bed nuclei of the stria terminalis	BST	0.566	0.035	0.058	0.536	0.033	0.063
Bed nucleus of the anterior commissure	BAC	-	-	-	-	-	-
Brain stem	BS	0.968	0.039	0.231	0.926	0.036	0.229
Interbrain	IB	0.927	0.055	0.223	0.889	0.051	0.219
Thalamus	TH	1.039	0.078	0.157	0.997	0.076	0.159
Thalamus sensory-motor cortex related	DORsm	1.067	0.076	0.133	1.030	0.074	0.137
Ventral group of the dorsal thalamus	VENT	1.077	0.080	0.113	1.040	0.079	0.114
Ventral anterior-lateral complex of the thalamus	VAL	1.126	0.088	0.067	1.089	0.085	0.065
Ventral medial nucleus of the thalamus	VM	1.004	0.082	0.055	0.962	0.083	0.054
Ventral posterior complex of the thalamus	VP	1.110	0.092	0.117	1.073	0.090	0.117

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Ventral posterolateral nucleus of the thalamus	VPL	1.076	0.106	0.056	1.043	0.112	0.054
Ventral posterolateral nucleus of the thalamus parvicellular part	VPLpc	-	-	-	-	-	-
Ventral posteromedial nucleus of the thalamus	VPM	1.158	0.093	0.112	1.120	0.094	0.111
Ventral posteromedial nucleus of the thalamus parvicellular part	VPMpc	-	-	-	-	-	-
Subparafascicular nucleus magnocellular part	SPFm	-	-	-	-	-	-
Subparafascicular nucleus parvicellular part	SPFp	-	-	-	-	-	-
Subparafascicular area	SPA	-	-	-	-	-	-
Peripeduncular nucleus	PP	-	-	-	-	-	-
Geniculate group dorsal thalamus	GENd	1.100	0.060	0.147	1.067	0.060	0.159
Medial geniculate complex	MG	1.028	0.097	0.150	0.990	0.108	0.159
Medial geniculate complex ventral part	MGv	1.057	0.136	0.156	1.021	0.150	0.150
Medial geniculate complex medial part	MGM	0.879	0.102	0.000	0.850	0.113	0.000
Dorsal part of the lateral geniculate complex	LGd	1.159	0.061	0.101	1.128	0.060	0.114
Dorsal part of the lateral geniculate complex core	LGd-co	1.116	0.105	0.094	1.084	0.108	0.103
Thalamus polymodal association cortex related	DORpm	1.052	0.085	0.160	1.007	0.082	0.161
Lateral group of the dorsal thalamus	LAT	0.985	0.088	0.123	0.947	0.086	0.126
Lateral posterior nucleus of the thalamus	LP	0.957	0.079	0.107	0.916	0.079	0.109
Posterior complex of the thalamus	PO	1.033	0.104	0.118	0.998	0.102	0.121
Posterior limiting nucleus of the thalamus	POL	-	-	-	-	-	-
Supragenulate nucleus	SGN	-	-	-	-	-	-
Anterior group of the dorsal thalamus	ATN	1.214	0.097	0.177	1.162	0.097	0.172
Anteroverentral nucleus of thalamus	AV	1.409	0.102	0.195	1.355	0.095	0.193
Anteromedial nucleus	AM	1.106	0.078	0.084	1.068	0.076	0.085
Anteromedial nucleus dorsal part	AMd	1.119	0.077	0.050	1.079	0.070	0.057
Anterodorsal nucleus	AD	-	-	-	-	-	-
Interanteromedial nucleus of the thalamus	IAM	-	-	-	-	-	-
Interanterodorsal nucleus of the thalamus	IAD	-	-	-	-	-	-
Lateral dorsal nucleus of thalamus	LD	1.162	0.094	0.133	1.107	0.096	0.130
Medial group of the dorsal thalamus	MED	1.057	0.093	0.126	1.018	0.090	0.128
Intermediodorsal nucleus of the thalamus	IMD	-	-	-	-	-	-
Mediodorsal nucleus of thalamus	MD	1.105	0.107	0.099	1.067	0.102	0.103
Submedial nucleus of the thalamus	SMT	0.981	0.070	0.086	0.933	0.075	0.082
Perireunensis nucleus	PR	-	-	-	-	-	-
Midline group of the dorsal thalamus	MTN	0.911	0.063	0.090	0.868	0.056	0.088
Paraventricular nucleus of the thalamus	PVT	-	-	-	-	-	-
Parataenial nucleus	PT	-	-	-	-	-	-
Nucleus of reuniens	RE	0.860	0.038	0.074	0.818	0.035	0.077
Intralaminar nuclei of the dorsal thalamus	ILM	1.086	0.096	0.123	1.041	0.095	0.126
Rhomboid nucleus	RH	-	-	-	-	-	-
Central medial nucleus of the thalamus	CM	-	-	-	-	-	-
Paracentral nucleus	PCN	-	-	-	-	-	-
Central lateral nucleus of the thalamus	CL	-	-	-	-	-	-
Parafascicular nucleus	PF	1.087	0.086	0.087	1.045	0.086	0.095
Reticular nucleus of the thalamus	RT	0.973	0.078	0.115	0.935	0.074	0.121
Geniculate group ventral thalamus	GENv	1.019	0.078	0.080	0.988	0.081	0.082
Intergeniculate leaflet of the lateral geniculate complex	IGL	-	-	-	-	-	-
Ventral part of the lateral geniculate complex	LGv	1.028	0.086	0.075	0.999	0.091	0.077
Subgeniculate nucleus	SubG	-	-	-	-	-	-
Epithalamus	EPI	1.111	0.092	0.220	1.001	0.091	0.256
Medial habenula	MH	1.076	0.161	0.101	0.874	0.039	0.082
Lateral habenula	LH	1.199	0.146	0.242	1.110	0.151	0.272
Hypothalamus	HY	0.768	0.034	0.213	0.736	0.030	0.208
Supraoptic nucleus	SO	-	-	-	-	-	-
Accessory supraoptic group	ASO	-	-	-	-	-	-
Paraventricular hypothalamic nucleus	PVH	-	-	-	-	-	-
Periventricular hypothalamic nucleus anterior part	PVa	-	-	-	-	-	-
Periventricular hypothalamic nucleus intermediate part	PVi	-	-	-	-	-	-
Arcuate hypothalamic nucleus	ARH	-	-	-	-	-	-

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Periventricular region	PVR	0.716	0.058	0.080	0.679	0.042	0.073
Anterodorsal preoptic nucleus	ADP	-	-	-	-	-	-
Anteroventral preoptic nucleus	AVP	-	-	-	-	-	-
Anteroventral periventricular nucleus	AVPV	-	-	-	-	-	-
Dorsomedial nucleus of the hypothalamus	DMH	0.678	0.052	0.045	0.640	0.037	0.048
Median preoptic nucleus	MEPO	-	-	-	-	-	-
Medial preoptic area	MPO	0.700	0.073	0.052	0.662	0.055	0.054
Vascular organ of the lamina terminalis	OV	-	-	-	-	-	-
Posterodorsal preoptic nucleus	PD	-	-	-	-	-	-
Parastrial nucleus	PS	-	-	-	-	-	-
Periventricular hypothalamic nucleus posterior part	PVp	-	-	-	-	-	-
Periventricular hypothalamic nucleus preoptic part	PVpo	-	-	-	-	-	-
Subparaventricular zone	SBPV	-	-	-	-	-	-
Suprachiasmatic nucleus	SCH	-	-	-	-	-	-
Ventrolateral preoptic nucleus	VLPO	-	-	-	-	-	-
Hypothalamic medial zone	MEZ	0.813	0.038	0.296	0.779	0.037	0.293
Anterior hypothalamic nucleus	AHN	0.699	0.051	0.058	0.675	0.046	0.063
Mammillary body	MBO	1.207	0.080	0.348	1.166	0.086	0.348
Lateral mammillary nucleus	LM	-	-	-	-	-	-
Medial mammillary nucleus	MM	1.426	0.080	0.312	1.386	0.088	0.312
Medial mammillary nucleus lateral part	Mml	1.229	0.211	0.139	1.198	0.212	0.142
Supramammillary nucleus	SUM	-	-	-	-	-	-
Tuberomammillary nucleus dorsal part	Tmd	-	-	-	-	-	-
Tuberomammillary nucleus ventral part	TMv	-	-	-	-	-	-
Medial preoptic nucleus	MPN	0.609	0.048	0.055	0.573	0.039	0.053
Dorsal preammillary nucleus	PMd	-	-	-	-	-	-
Ventral preammillary nucleus	PMv	0.546	0.073	0.053	0.525	0.067	0.047
Paraventricular hypothalamic nucleus descending division	PVHd	-	-	-	-	-	-
Ventromedial hypothalamic nucleus	VMH	0.630	0.022	0.056	0.606	0.022	0.061
Posterior hypothalamic nucleus	PH	0.747	0.050	0.067	0.707	0.042	0.068
Hypothalamic lateral zone	LZ	0.854	0.032	0.232	0.823	0.029	0.225
Lateral hypothalamic area	LHA	0.737	0.035	0.075	0.710	0.032	0.072
Lateral preoptic area	LPO	0.728	0.044	0.072	0.706	0.036	0.070
Preparasubthalamic nucleus	PST	-	-	-	-	-	-
Parasubthalamic nucleus	PSTN	-	-	-	-	-	-
Retrochiasmatic area	RCH	-	-	-	-	-	-
Subthalamic nucleus	STN	-	-	-	-	-	-
Tuberal nucleus	TU	0.603	0.058	0.039	0.582	0.061	0.041
Zona incerta	ZI	1.155	0.095	0.178	1.113	0.091	0.178
Dopaminergic A13 group	A13	-	-	-	-	-	-
Fields of Forel	FF	1.018	0.097	0.053	0.978	0.089	0.049
Midbrain	MB	0.970	0.055	0.235	0.926	0.051	0.234
Midbrain sensory related	MBsen	1.310	0.040	0.323	1.257	0.039	0.333
Superior colliculus sensory related	SCs	0.953	0.056	0.082	0.888	0.059	0.097
Superior colliculus optic layer	SCop	-	-	-	-	-	-
Superior colliculus superficial gray layer	SCsg	-	-	-	-	-	-
Inferior colliculus	IC	1.455	0.044	0.265	1.409	0.041	0.267
Inferior colliculus central nucleus	ICc	1.754	0.098	0.122	1.709	0.092	0.126
Inferior colliculus dorsal nucleus	ICd	1.288	0.055	0.186	1.244	0.054	0.186
Inferior colliculus external nucleus	ICe	1.243	0.031	0.189	1.194	0.039	0.203
Nucleus of the brachium of the inferior colliculus	NB	-	-	-	-	-	-
Nucleus sagulum	SAG	-	-	-	-	-	-
Parabigeminal nucleus	PBG	-	-	-	-	-	-
Midbrain trigeminal nucleus	MEV	-	-	-	-	-	-
Midbrain motor related	MBmot	0.931	0.063	0.165	0.891	0.057	0.162
Substantia nigra reticular part	SNr	0.826	0.031	0.140	0.791	0.028	0.138
Ventral tegmental area	VTA	0.790	0.044	0.056	0.755	0.034	0.061
Midbrain reticular nucleus retrorubral area	RR	-	-	-	-	-	-
Midbrain reticular nucleus	MRN	0.884	0.059	0.111	0.847	0.056	0.110
Superior colliculus motor related	SCm	0.985	0.057	0.131	0.945	0.051	0.129
Superior colliculus motor related deep gray layer	SCdg	0.905	0.052	0.068	0.876	0.049	0.066
Superior colliculus motor related deep white layer	SCdw	-	-	-	-	-	-

Table 4.2 Regional vessel length densities. Computed from the densities in 240-cubes in the region. Small regions without enough 240-cubes are shown as “-”. (Continue)

Superior colliculus motor related intermediate white layer	SCiw	1.048	0.090	0.100	1.008	0.083	0.099
Superior colliculus motor related intermediate gray layer	SCig	1.037	0.054	0.155	0.990	0.052	0.154
Superior colliculus motor related intermediate gray layer sublayer a	SCig-a	-	-	-	-	-	-
Superior colliculus motor related intermediate gray layer sublayer b	SCig-b	-	-	-	-	-	-
Superior colliculus motor related intermediate gray layer sublayer c	SCig-c	-	-	-	-	-	-
Periaqueductal gray	PAG	0.811	0.076	0.162	0.772	0.068	0.157
Precommissural nucleus	PRC	0.784	0.093	0.078	0.740	0.093	0.074
Interstitial nucleus of Cajal	INC	-	-	-	-	-	-
Nucleus of Darkschewitsch	ND	-	-	-	-	-	-
Pretecal region	PRT	1.119	0.094	0.149	1.068	0.089	0.149
Anterior pretecal nucleus	APN	1.191	0.098	0.119	1.138	0.094	0.124
Medial pretecal area	MPT	-	-	-	-	-	-
Nucleus of the optic tract	NOT	-	-	-	-	-	-
Nucleus of the posterior commissure	NPC	0.927	0.096	0.076	0.900	0.084	0.083
Olivary pretecal nucleus	OP	-	-	-	-	-	-
Posterior pretecal nucleus	PPT	-	-	-	-	-	-
Cuneiform nucleus	CUN	0.889	0.088	0.109	0.860	0.084	0.109
Red nucleus	RN	1.090	0.111	0.125	1.045	0.106	0.123
Oculomotor nucleus	III	-	-	-	-	-	-
Edinger-Westphal nucleus	EW	-	-	-	-	-	-
Trochlear nucleus	IV	-	-	-	-	-	-
Ventral tegmental nucleus	VTN	-	-	-	-	-	-
Anterior tegmental nucleus	AT	-	-	-	-	-	-
Lateral terminal nucleus of the accessory optic tract	LT	-	-	-	-	-	-
Midbrain behavioral state related	MBsta	0.843	0.059	0.142	0.795	0.063	0.131
Substantia nigra compact part	SNc	-	-	-	-	-	-
Pedunculopontine nucleus	PPN	0.793	0.047	0.084	0.763	0.047	0.081
Midbrain raphe nuclei	RAmb	0.979	0.144	0.159	0.877	0.165	0.168
Interfascicular nucleus raphe	IF	-	-	-	-	-	-
Interpeduncular nucleus	IPN	1.026	0.111	0.134	0.921	0.135	0.151
Rostral linear nucleus raphe	RL	-	-	-	-	-	-
Central linear nucleus raphe	CLI	-	-	-	-	-	-
Dorsal nucleus raphe	DR	-	-	-	-	-	-
Hindbrain	HB	1.009	0.021	0.230	0.964	0.019	0.228
Pons	P	0.968	0.036	0.223	0.925	0.034	0.223
Pons sensory related	P-sen	1.175	0.025	0.301	1.132	0.024	0.298
Nucleus of the lateral lemniscus	NLL	1.275	0.143	0.234	1.218	0.137	0.238
Principal sensory nucleus of the trigeminal	PSV	1.167	0.046	0.204	1.128	0.045	0.206
Parabrachial nucleus	PB	0.815	0.048	0.101	0.779	0.048	0.099
Koelliker-Fuse subnucleus	KF	-	-	-	-	-	-
Superior olivary complex	SOC	1.419	0.041	0.242	1.373	0.040	0.242
Superior olivary complex periolivary region	POR	1.103	0.096	0.098	1.061	0.094	0.098
Superior olivary complex medial part	SOCm	-	-	-	-	-	-
Superior olivary complex lateral part	SOCI	1.614	0.141	0.191	1.562	0.137	0.192
Pons motor related	P-mot	0.970	0.041	0.200	0.930	0.037	0.196
Barrington's nucleus	B	-	-	-	-	-	-
Dorsal tegmental nucleus	DTN	-	-	-	-	-	-
Pontine central gray	PCG	1.091	0.104	0.169	1.035	0.093	0.168
Pontine gray	PG	1.141	0.094	0.205	1.084	0.088	0.228
Pontine reticular nucleus caudal part	PRNc	0.850	0.030	0.074	0.816	0.029	0.073
Supragenua nucleus	SG	-	-	-	-	-	-
Supratrigeminal nucleus	SUT	-	-	-	-	-	-
Tegmental reticular nucleus	TRN	1.032	0.036	0.193	1.003	0.038	0.187
Motor nucleus of trigeminal	V	1.113	0.055	0.099	1.069	0.055	0.111
Peritrigeminal zone	P5	0.941	0.069	0.090	0.904	0.065	0.088
Accessory trigeminal nucleus	Acs5	-	-	-	-	-	-
Pons behavioral state related	P-sat	0.868	0.049	0.099	0.825	0.048	0.099
Superior central nucleus raphe	CS	0.969	0.086	0.069	0.917	0.076	0.067
Locus ceruleus	LC	-	-	-	-	-	-
Laterodorsal tegmental nucleus	LDT	0.968	0.082	0.039	0.882	0.083	0.031
Nucleus incertus	NI	-	-	-	-	-	-
Pontine reticular nucleus	PRNr	0.850	0.045	0.091	0.810	0.045	0.094
Nucleus raphe pontis	RPO	-	-	-	-	-	-

Table 4.2 Regional vessel length densities. Computed from the densities in 240-cubes in the region. Small regions without enough 240-cubes are shown as “-“. (Continue)

Subceruleus nucleus	SLC	-	-	-	-	-	-
Sublaterodorsal nucleus	SLD	-	-	-	-	-	-
Medulla	MY	1.026	0.018	0.229	0.981	0.017	0.226
Medulla sensory related	MY-sen	1.124	0.012	0.235	1.080	0.012	0.235
Area postrema	AP	-	-	-	-	-	-
Cochlear nuclei	CN	1.549	0.045	0.193	1.487	0.044	0.204
Dorsal cochlear nucleus	DCO	1.462	0.077	0.180	1.385	0.068	0.181
Ventral cochlear nucleus	VCO	1.622	0.106	0.180	1.572	0.106	0.190
Cuneate nucleus	CU	-	-	-	-	-	-
Gracile nucleus	GR	-	-	-	-	-	-
External cuneate nucleus	ECU	-	-	-	-	-	-
Nucleus of the trapezoid body	NTB	-	-	-	-	-	-
Nucleus of the solitary tract	NTS	-	-	-	-	-	-
Spinal nucleus of the trigeminal caudal part	SPVC	1.059	0.035	0.136	1.024	0.038	0.138
Spinal nucleus of the trigeminal interpolar part	SPVI	1.080	0.046	0.186	1.038	0.045	0.193
Spinal nucleus of the trigeminal oral part	SPVO	1.114	0.031	0.116	1.080	0.028	0.115
Medulla motor related	MY-mot	1.017	0.025	0.221	0.973	0.025	0.217
Abducens nucleus	VI	-	-	-	-	-	-
Accessory abducens nucleus	ACVI	-	-	-	-	-	-
Facial motor nucleus	VII	1.095	0.031	0.138	1.055	0.029	0.139
Accessory facial motor nucleus	ACVII	-	-	-	-	-	-
Nucleus ambiguus dorsal division	AMBd	-	-	-	-	-	-
Nucleus ambiguus ventral division	AMBv	-	-	-	-	-	-
Dorsal motor nucleus of the vagus nerve	DMX	-	-	-	-	-	-
Gigantocellular reticular nucleus	GRN	0.904	0.037	0.072	0.869	0.037	0.072
Infracerebellar nucleus	ICB	-	-	-	-	-	-
Inferior olivary complex	IO	-	-	-	-	-	-
Intermediate reticular nucleus	IRN	0.923	0.037	0.081	0.880	0.036	0.080
Inferior salivatory nucleus	ISN	-	-	-	-	-	-
Linear nucleus of the medulla	LIN	-	-	-	-	-	-
Lateral reticular nucleus	LRN	0.999	0.038	0.117	0.948	0.037	0.121
Lateral reticular nucleus magnocellular part	LRNm	1.005	0.031	0.113	0.955	0.030	0.116
Magnocellular reticular nucleus	MARN	0.887	0.037	0.058	0.857	0.030	0.060
Medullary reticular nucleus	MDRN	0.899	0.010	0.089	0.857	0.011	0.089
Medullary reticular nucleus dorsal part	MDRNd	0.880	0.018	0.090	0.840	0.018	0.090
Medullary reticular nucleus ventral part	MDRNv	0.917	0.023	0.077	0.875	0.018	0.077
Parvicellular reticular nucleus	PARN	0.911	0.040	0.077	0.861	0.040	0.081
Parasolitary nucleus	PAS	-	-	-	-	-	-
Paragigantocellular reticular nucleus	PGRN	0.976	0.042	0.085	0.933	0.042	0.087
Paragigantocellular reticular nucleus dorsal part	PGRNd	1.090	0.077	0.020	1.049	0.075	0.025
Paragigantocellular reticular nucleus lateral part	PGRNI	0.954	0.040	0.067	0.910	0.038	0.070
Nucleus of Roller	NR	-	-	-	-	-	-
Nucleus prepositus	PRP	-	-	-	-	-	-
Parapyramidal nucleus	PPY	-	-	-	-	-	-
Vestibular nuclei	VNC	1.402	0.057	0.187	1.350	0.055	0.184
Lateral vestibular nucleus	LAV	1.019	0.194	0.088	0.983	0.191	0.090
Medial vestibular nucleus	MV	1.475	0.076	0.161	1.414	0.071	0.160
Spinal vestibular nucleus	SPIV	1.325	0.034	0.143	1.284	0.036	0.147
Superior vestibular nucleus	SUV	1.400	0.119	0.113	1.364	0.126	0.120
Nucleus x	x	-	-	-	-	-	-
Hypoglossal nucleus	XII	1.030	0.055	0.091	0.979	0.060	0.101
Nucleus y	y	-	-	-	-	-	-
Nucleus raphe magnus	RM	-	-	-	-	-	-
Nucleus raphe pallidus	RPA	-	-	-	-	-	-
Nucleus raphe obscurus	RO	-	-	-	-	-	-
Cerebellum	CB	1.096	0.045	0.161	1.056	0.041	0.169
Cerebellar cortex	CBX	1.081	0.046	0.135	1.042	0.041	0.146
Vermal regions	VERM	1.119	0.056	0.145	1.073	0.051	0.160
Lingula (I)	LING	-	-	-	-	-	-
Central lobule	CENT	1.168	0.091	0.178	1.106	0.080	0.210
Lobule II	CENT2	1.156	0.122	0.207	1.085	0.108	0.245
Lobule III	CENT3	1.183	0.090	0.157	1.132	0.082	0.181
Culmen	CUL	1.118	0.045	0.124	1.079	0.039	0.142
Lobules IV-V	CUL4 5	1.118	0.045	0.124	1.079	0.039	0.142
Declive (VI)	DEC	1.075	0.032	0.099	1.043	0.027	0.114
Folium-tuber vermis (VII)	FOTU	1.036	0.040	0.100	1.003	0.041	0.118

Table 4.2 Regional vessel length densities. Computed from the densities in 240-cubes in the region. Small regions without enough 240-cubes are shown as “-“. (Continue)

Pyramus (VIII)	PYR	1.133	0.029	0.108	1.108	0.027	0.113
Uvula (IX)	UVU	1.164	0.044	0.112	1.129	0.044	0.126
Nodulus (X)	NOD	1.307	0.116	0.114	1.226	0.095	0.110
Hemispheric regions	HEM	1.050	0.043	0.112	1.018	0.040	0.124
Simple lobule	SIM	1.082	0.035	0.090	1.057	0.033	0.100
Ansiform lobule	AN	1.052	0.048	0.109	1.020	0.045	0.123
Crus 1	ANcr1	1.067	0.042	0.096	1.037	0.040	0.108
Crus 2	ANcr2	1.072	0.054	0.101	1.043	0.051	0.112
Paramedian lobule	PRM	1.052	0.036	0.088	1.026	0.032	0.096
Copula pyramidis	COPY	1.031	0.066	0.108	1.002	0.067	0.121
Paraflocculus	PFL	-	-	-	-	-	-
Flocculus	FL	-	-	-	-	-	-
Cerebellar nuclei	CBN	1.496	0.088	0.210	1.450	0.084	0.208
Fastigial nucleus	FN	1.371	0.069	0.113	1.325	0.064	0.116
Interposed nucleus	IP	1.571	0.099	0.179	1.520	0.094	0.180
Dentate nucleus	DN	1.485	0.117	0.241	1.447	0.113	0.243

Table 4.3. Regional name and abbreviation

Abbreviation	Structure name	Abbreviation	Structure name
ACA	Anterior cingulate area	MO	Somatomotor areas
AI	Agranular insular area	MOB	Main olfactory bulb
AOB	Accessory olfactory bulb	MY	Medulla
AON	Anterior olfactory nucleus	NLOT	Nucleus of the lateral olfactory tract
AUD	Auditory areas	ORB	Orbital area
CA	Ammon's horn	P	Pons
CBN	Cerebellar nuclei	PAA	Piriform-amygdalar area
CBX	Cerebellar cortex	PAL	Pallidum
cc	corpus callosum	PERI	Perirhinal area
COA	Cortical amygdalar area	PIR	Piriform area
CTXsp	Cortical subplate	PL	Prelimbic area
DG	Dentate gyrus	PTLp	Posterior parietal association areas
DP	Dorsal peduncular area	RHP	Retrohippocampal region
ECT	Ectorhinal area	RSP	Retrosplenial area
FC	Fasciola cinerea	SS	Somatosensory areas
FRP	Frontal pole cerebral cortex	STR	Striatum
GU	Gustatory areas	TEa	Temporal association areas
HY	Hypothalamus	TH	Thalamus
IG	Induseum griseum	TR	Postpiriform transition area
ILA	Infralimbic area	TT	Taenia tecta
MBmot	Midbrain motor related	VIS	Visual areas
MBsen	Midbrain sensory related	VISC	Visceral area
MBsta	Midbrain behavioral state related		

Table 4.4. Data for generating Figure 4.20

Region Name	Glucose metabolism rate (M/s)		Vessel length density (m/mm ³)						Average dmax (µm)						Number of cubes		
	Mean	SD	25 percentile			75 percentile			25 percentile			75 percentile					
			Brain1	Brain2	Brain3	Brain1	Brain2	Brain3	Brain1	Brain2	Brain3	Brain1	Brain2	Brain3	Brain1	Brain2	Brain3
Internal capsule	6.77E-06	6.08E-07	0.65	0.51	0.65	0.58	0.46	0.60	0.69	0.56	0.78	37.4	41.4	36.4	32.8	39.0	38.0
	7.77E-06	3.99E-07	0.92	0.77	0.82	0.82	0.68	0.75	1.00	0.84	0.91	30.9	33.8	32.6	29.1	32.0	30.7
	8.34E-06	5.73E-07	0.55	0.36	0.59	0.50	0.28	0.52	0.63	0.41	0.65	38.8	48.8	38.5	36.3	45.2	35.8
	9.73E-06	4.52E-07	0.95	0.86	0.82	0.87	0.77	0.75	1.06	0.93	0.90	29.3	30.5	30.6	27.5	29.1	29.4
	9.90E-06	4.69E-07	0.72	0.70	0.66	0.65	0.63	0.60	0.80	0.78	0.74	34.2	34.7	35.3	32.2	32.8	37.4
Hypothalamus	1.04E-05	8.68E-07	1.01	0.89	0.95	0.91	0.81	0.88	1.09	0.93	0.99	28.3	30.1	29.0	27.1	29.3	28.3
	1.06E-05	5.38E-07	0.76	0.67	0.72	0.68	0.60	0.65	0.85	0.76	0.82	33.5	35.7	33.9	31.4	33.4	31.8
Lateral septal nucleus	1.06E-05	7.47E-07	0.71	0.59	0.64	0.63	0.48	0.57	0.75	0.64	0.69	34.6	37.9	36.3	33.3	36.0	34.3
	1.06E-05	6.60E-07	0.68	0.70	0.70	0.59	0.62	0.63	0.82	0.83	0.83	34.8	33.8	34.1	31.3	30.8	31.3
Piriform cortex	1.06E-05	5.73E-07	0.85	0.81	0.79	0.78	0.74	0.71	0.93	0.92	0.88	30.8	31.6	31.8	29.4	30.0	30.3
	1.06E-05	6.08E-07	0.93	0.95	0.89	0.87	0.88	0.82	1.02	1.04	0.99	29.3	29.0	29.6	27.9	27.7	28.0
Substantia nigra	1.15E-05	7.29E-07	0.73	0.74	0.77	0.66	0.68	0.71	0.81	0.82	0.85	32.9	32.8	32.1	31.0	30.8	30.6
	1.29E-05	5.21E-07	1.25	1.04	0.97	1.16	0.97	0.87	1.36	1.14	1.06	25.8	27.3	28.2	24.8	26.3	27.3
Caudate nucleus	1.32E-05	5.04E-07	0.91	0.84	0.83	0.84	0.76	0.77	0.97	0.91	0.90	30.2	31.4	31.3	29.0	29.8	30.1
	1.37E-05	5.90E-07	0.97	0.93	0.87	0.86	0.84	0.79	1.14	1.13	1.03	28.9	29.4	30.1	26.6	26.7	27.8
Occipital cortex	1.48E-05	4.86E-07	0.86	0.84	0.87	0.78	0.77	0.79	0.93	0.92	0.93	30.0	30.2	29.9	28.7	28.6	31.8
	1.48E-05	5.38E-07	1.19	1.15	1.04	1.10	0.95	1.00	1.27	1.22	1.12	26.1	26.8	27.3	25.1	25.2	26.1
Ventral nucleus (Thalamus)	1.51E-05	6.95E-07	1.18	1.03	1.00	1.11	0.96	0.95	1.26	1.13	1.07	26.5	28.0	28.1	25.3	26.4	27.0
	1.58E-05	4.86E-07	1.01	0.91	0.93	0.95	0.85	0.86	1.12	1.00	1.00	28.1	29.3	29.1	26.6	27.8	28.1
Superior colliculus	1.58E-05	5.56E-07	0.92	0.86	0.89	0.86	0.80	0.82	0.97	0.95	0.95	29.2	30.3	29.8	28.3	28.8	30.3
	1.60E-05	7.64E-07	1.13	1.09	1.00	1.02	0.95	0.92	1.23	1.17	1.09	26.7	27.0	27.6	25.4	25.7	26.3
Medial geniculate (Thalamus)	1.62E-05	5.04E-07	1.04	1.02	1.02	0.93	0.88	0.91	1.14	1.13	1.12	27.2	27.3	27.5	26.0	25.9	26.2
	1.62E-05	5.04E-07	1.28	1.13	1.09	1.20	1.03	1.03	1.38	1.20	1.17	25.1	26.7	27.1	24.0	25.8	26.0
Anterior nucleus (Thalamus)	1.70E-05	8.51E-07	1.56	1.53	1.58	1.46	1.38	1.39	1.65	1.73	1.73	22.6	22.5	22.5	20.8	21.0	23.4
	1.74E-05	7.12E-07	1.45	1.48	1.40	1.18	1.32	1.28	1.61	1.58	1.53	23.3	22.9	23.6	22.3	22.4	22.3
Cochlear nucleus	1.81E-05	6.08E-07	1.44	1.45	1.35	1.29	1.30	1.22	1.57	1.58	1.44	23.4	23.5	24.0	22.5	22.3	23.0
	1.88E-05	7.29E-07	1.50	1.49	1.45	1.27	1.28	1.29	1.75	1.70	1.57	22.4	22.7	22.8	20.7	21.0	21.8
Vestibular nucleus	2.22E-05	7.29E-07	1.50	1.49	1.45	1.27	1.28	1.29	1.75	1.70	1.57	22.4	22.7	22.8	20.7	21.0	21.8
	2.22E-05	7.29E-07	1.50	1.49	1.45	1.27	1.28	1.29	1.75	1.70	1.57	22.4	22.7	22.8	20.7	21.0	21.8
Inferior colliculus	2.22E-05	7.29E-07	1.50	1.49	1.45	1.27	1.28	1.29	1.75	1.70	1.57	22.4	22.7	22.8	20.7	21.0	21.8
	2.22E-05	7.29E-07	1.50	1.49	1.45	1.27	1.28	1.29	1.75	1.70	1.57	22.4	22.7	22.8	20.7	21.0	21.8

Table 4.5. Extra plot data for Figure 4.21

Species	Region	Length density (m/mm3)	Density reference	kO2 ($\mu\text{mol}/(100\text{g} \cdot \text{min})$)	Metabolism references	Radius (μm)	Radius reference
Macaque	Visual cortex	0.478	Weber et al., 2008	325	Kennedy et al., 1978	2.75	Weber et al., 2008
Human	Primary motor cortex	0.499	Cassot et al., 2006	185	Vafaee et al., 2012	3	Cassot et al., 2006
Human	Frontal cortex	0.363	ernandez-Klett et al., 202	181	Ishii et al., 1996; Karbowski, 2014; Kuhl et al., 1982; Pantano et al., 1984	3	Cassot et al., 2006
Human	Thalamus	0.281	Kubikova et al., 2018	183	Pantano et al., 1984; Kuhl et al., 1982; Heiss et al., 1984; Hatazawa et al., 1995	3	Cassot et al., 2006
Human	Pons	0.291	Kubikova et al., 2018	120	Heiss et al., 1984; Hatazawa et al., 1995	3	Cassot et al., 2006
Human	White matter	0.160	Kubikova et al., 2018	90	Pantano et al., 1984; Heiss et al., 1984; Hatazawa et al., 1995	3	Cassot et al., 2006
Human	Cerebellum cortex	0.296	Kubikova et al., 2018	179	Tomasi et al., 2013; Heiss et al., 1984; Hatazawa et al., 1995	3	Cassot et al., 2006
Human	Caudateputamen	0.347	Kubikova et al., 2018	210	Kuhl et al., 1982; Heiss et al., 1984; Hatazawa et al., 1995	3	Cassot et al., 2006
				Average over all references			

Chapter 5

All optical histology of brains in their cranium

Fresh blood enters the brain vascular network primarily through two arterial sources: the carotid and vertebral arteries. The internal carotid arteries and the vertebral arteries traverse cranial foramina and foramen magnum, respectively, to reach the brain. Concomitantly, a series of major veins drain the deoxygenated blood away from the brain into the venous sinuses. These venous sinuses exhibit numerous connections with the skull marrow cavities, which are recently found to be connected to meningeal vessels and facilitate brain immune response[13, 44]. Consequently, a complete picture of the vasculature should include not only the hierarchical network within the brain parenchyma but also the vascular components in the meninges and the skull.

The multi-scale nature of the network poses numerous technical challenges in acquiring such a complete picture. Even for rodents, the head vasculature nourishes the entire tissue on centimeter scale, while the finest capillaries have caliber down to micrometer. To clearly resolve and quantify all the fine connections, a centimeter-scaled heterogeneous sample requires sub-micrometer imaging. Recent advances in tissue cleaning and light-sheet microscopy have enabled rapid imaging of almost transparent tissue on scale, revealing the gross structure and leading to the co-discovery of the skull-meninges connections. However, scattering in an imperfectly cleared sample substantially deteriorates the optical resolution and signal-to-noise level deep

in the tissue. This becomes more severe when high numerical aperture objectives are used while the skull and meninges add extra difficulties in index matching. Alternatively, serial two-photon microscopy used a vibratome to physically remove part of the imaged layer, ensuring a constant imaging quality through the sample. However, successful automatic sectioning requires relatively homogeneous samples with uniform resistance. The mechanical properties of skull and meninges membranes appear to be incompatible with a conventional vibratome. Therefore, for high-resolution imaging of heterogeneous biological tissue, we seek an alternative tissue removal approach to the use of a vibratome in serial two-photon microscopy.

5.1 Simultaneous spatiotemporal focusing for efficient under liquid ablation

Plasma-mediated laser ablation has been used to remove material of distinct mechanical properties with high spatial precision. Tightly focused ultrafast pulses produce highly localized plasma through nonlinear absorption processes and ablate the targeted material. Due to the ultrafast dynamic of this process, heat transfer to the surrounding matter is minimized and so does the collateral damage. Specifically for biological tissues, past works have demonstrated femtosecond laser ablation on neuronal tissues ([73, 120]), mouse skull ([51, 83]), porcine cornea ([91]), etc. All these works ablate one relatively homogeneous material with a focused Gaussian beam, and most of them only demonstrated feasibility on a scale of (sub)-millimeter.

Serial imaging of a mouse head requires ablation of the imaged tissue layer of thickness about 100 μm and lateral extend of a few square millimeters. In our setup, tissue ablation is the rate-limiting step and therefore increasing ablation efficiency is crucial. In the past, plasma-mediated ablation of neuronal tissue and skull was achieved by focusing a Gaussian beam and therefore the lateral and axial extend of the focal volume is coupled ([120, 51]). As femtosecond laser ablation depends on multiphoton ionization to initialize plasma formation, the peak photon fluence is crucial for achieving efficient ablation. Therefore, we applied simultaneous

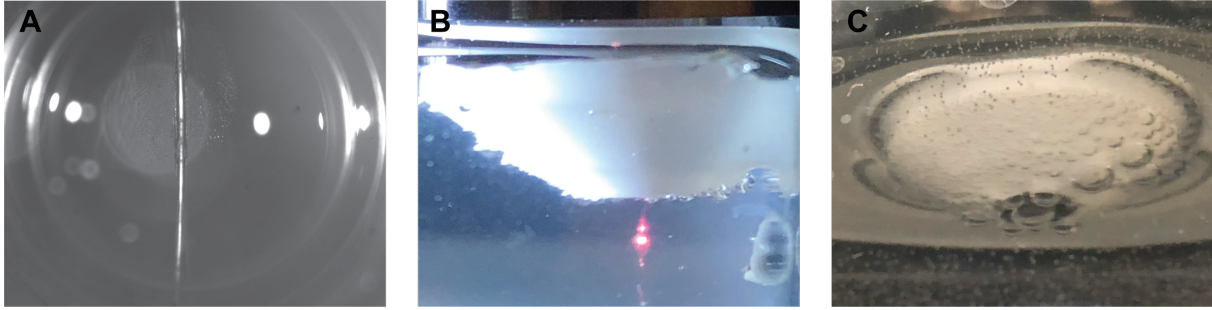


Figure 5.1. Problems in under liquid ablation using simultaneous spatiotemporal focusing. (A) Objective damaged by the ablation beam. This objective has a 2 mm working distance, 0.95 NA. The damage (central vertical line) appears to be near the front aperture of the objective. (B) Under-water femtosecond laser ablation produces gas bubbles. (C) Gas bubbles block the front aperture of the objective and interrupt the ablation process.

spatiotemporal focusing (SSTP) to extend the later ablation area while confining the axial extend. SSTP uses a diffraction grating to spatially separate different frequency components of an ultrafast pulse and only recombine these components at the focus of the objective lens ([137, 92]). As a result, the effective pulse width is stretched by typically tens of times, and the nonlinear interactions out of focus are suppressed. This technique has been applied to low NA ablation of materials and shown to improve ablation precision by suppressing self-focusing that shifts the focus toward the objective ([55, 7]).

Based on our previous design ([120]), we used the same high numerical aperture (NA) objective for both imaging and ablation in a liquid environment. The ablation module consisted of a Ti:Sapphire ultrafast laser amplifier (Astrella, Coherent, USA) running at 1 kHz and 5 W with a pulse width of about 100 fs. The output beam of 11 mm in diameter ($1/e^2$) is passed through a half waveplate mounted on a motorized rotational stage and split into two arms by a high-power polarizing beam splitter (PBS25-780-HP, Thorlabs, USA). The S-polarized component is directed onto a reflective diffraction grating (830/mm, 53107ZD02-035R, Richardson Gratings, USA) at 41.61° so that the principle diffracted beam propagates perpendicularly to the grating surface. This configuration facilitates the control of the lateral extension of the ablation volume.

5.1.1 Accumulated heat damaged objective

We first experimented with a plane ablation pattern. A 750 mm focal length achromatic spherical lens was used along with the objective in the 4f configuration. This setup produces a 5.77 mm ($1/e^2$ diameter) wide line focusing pattern at the back aperture of the objective and a $139 \times 104 \mu\text{m}^2$ ablation disk on the focal plane. Consequently, the Pulse Width Stretch Factor (PWSF) is about 41 ([137]). However, the achromatic lens focuses the diffracted beam into a line at the back aperture of the objective. We first tested this ablation scheme with a 20x 0.95 NA objective (XLUMPlanFI, Olympus, Japan). Though the pulse width has been stretched and the pulse energy is insufficient to produce nonlinear breakdown in transparent material, such as the glasses and the optical glue that made up the objective, we observed damage near the front optical aperture after minutes of ablation (Figure 5.1A). This damage is probably due to accumulated heat as the achromatic lens produced an intense line focus inside the objective and about 40% of the laser power (at 800 nm) is absorbed by the objective[112]. To protect the objective, a 0.5° (FWHM) optical diffuser was added right after the grating to decrease the fluence inside the objective by about 60 times. Given the long focal length of the achromatic lens and the small diffraction angle of the diffuser, the maximum pulse broadening due to the diffuse is about 100 femtosecond and therefore the temporal stretching and refocusing is dominantly contributed by the grating. We have also explored using a diffuser alone for temporal focusing. Despite having a much smaller PWSF (≈ 2), the spatial pattern of a practical optical diffuser gave rise to a local intensity peak at the back aperture of the objective. This uneven distribution of light intensity, combined with the high absorption rate of the objective, produced pepper-like damage near the front aperture of the objective after heat accumulation.

5.1.2 Gas bubble generation

Under liquid femtosecond laser ablation ionized molecules and produce cavitation bubbles (Figure 5.1B). The size, lifetime, and chemical compositions of the gas bubble depend on

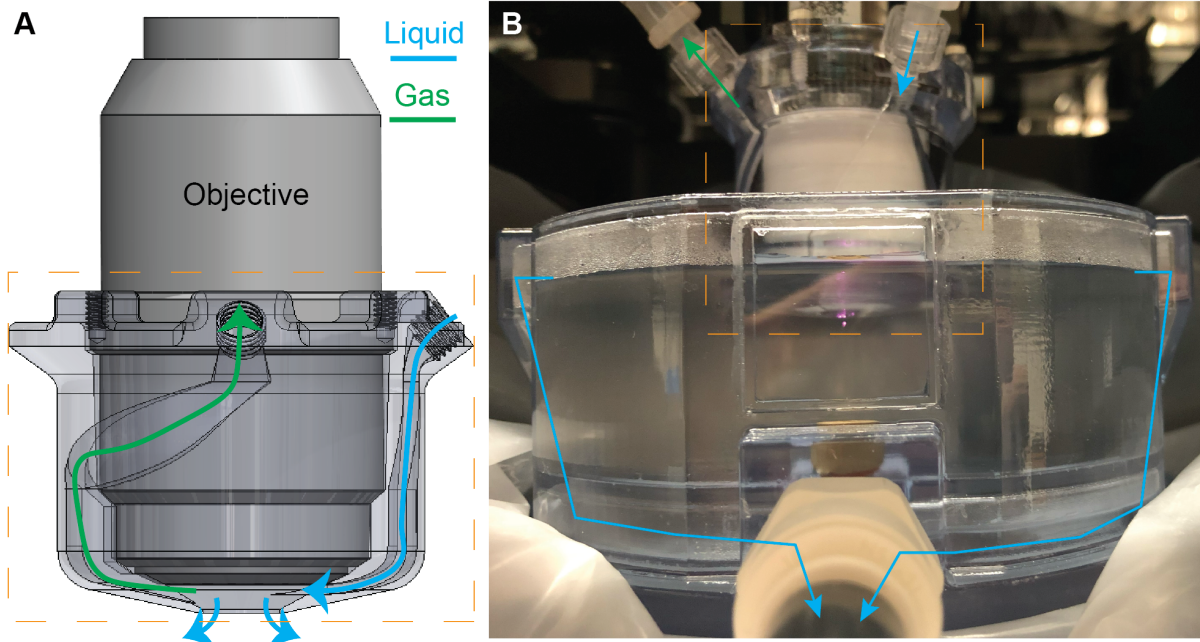


Figure 5.2. Flow system for gas bubble removal. (A) 3D model of the objective with the customized cap for gas bubble removal. (B) Flow system under operation. Blue lines indicate the liquid flow direction. Green lines show the gas flow direction.

laser fluence and liquid physical and chemical properties[116]. Some of the cavitation bubbles rise while others merge and stick to the surface of the ablation target for seconds, interfering with the subsequent ablation pulses [54]. For high-NA under liquid ablation, the rising bubbles accumulated under the objective front aperture. Long-duration ablation processes produced a significant amount of bubbles that could completely interrupt the ablation process (Figure 5.1C).

To prevent gas bubbles from blocking the front aperture of the objective, and to remove the persistent bubbles on the surface of the sample, we designed and implemented a closed-loop flow system with an open surface. Liquid in the reservoir is gravity-fed into a magnetic-driven pump (1/50 HP, 99845K46, McMaster-Carr, USA) and pushed through an inline filter (5 μm , 1 GPM, PENTAIR/PENTEK 255575-75, Grainger, USA) before passing through the check valves and an adjustable flow meter. The flow is then channeled into a custom-designed objective cap that flushes the front aperture of the objective and the surface of the ablation target (Figure 5.2A). The sample is mounted in a custom-designed holder that drains the excessive liquid back to the

reservoir (Figure 5.2B). Both the objective cap and sample holder were manufactured using Stereolithography (made of Accura ClearVue, with Quick Clear finishing, Xometry, USA). For operating with 60% DMSO 40% water solution (detailed in subsection 5.2.1), about 10 Gallon per hour is needed to remove the persistent bubbles. In addition, a high flow rate is required to suppress biofilm formation on the optical front aperture of the objective.

5.1.3 Efficient ablation on fluorescent gelatin block

These technical improvements overcome the challenges of high-NA under-liquid femtosecond laser ablation over extended periods. Ensuring the objective lens was protected and bubble interference eliminated, we experimented on ablation with fluorescent gelatin blocks utilizing a planar ablation geometry. The block, comprising 10% gelatin by volume with 0.4% Fluorescein isothiocyanate (FITC) in phosphate-buffered saline (PBS), was moved across the objective at 70 mm/s. After each ablation line, the stage along the orthogonal direction stepped by 73 μm to initiate a new line of ablation. With spatiotemporally focused femtosecond pulses, we achieved efficient material removal, excising a volume of $700 \times 700 \times 50 \mu\text{m}^3$ with an ablation fluence of 7 J/cm^2 and an efficiency of $5.11 \times 10^7 \mu\text{m}^3/\text{s}$. Post ablation, the targeted region was repositioned away from the imaging field and imaged with two-photon microscopy (Figure 5.3). This process produced a sharp boundary between the ablated and intact area laterally. Despite the later precision, the ablated surface exhibited fragmentation approximately 10 μm beneath the final ablation plane(Figure 5.3C), although the bulk of material removal was confined within 10 μm of the last ablated layer. Surprisingly, some gelatin damage extended beyond 50 μm from the final ablation plane(Figure 5.3D).

5.2 Optimize ablation for heterogeneous biological tissues

Serial two-photon microscopy images an extended-sized sample by iterating imaging and material removal. The imaged volume before and after sample removal should have sufficient overlap (30 μm to 50 μm) for subsequent image stitching. The imaging depth of each 3D volume

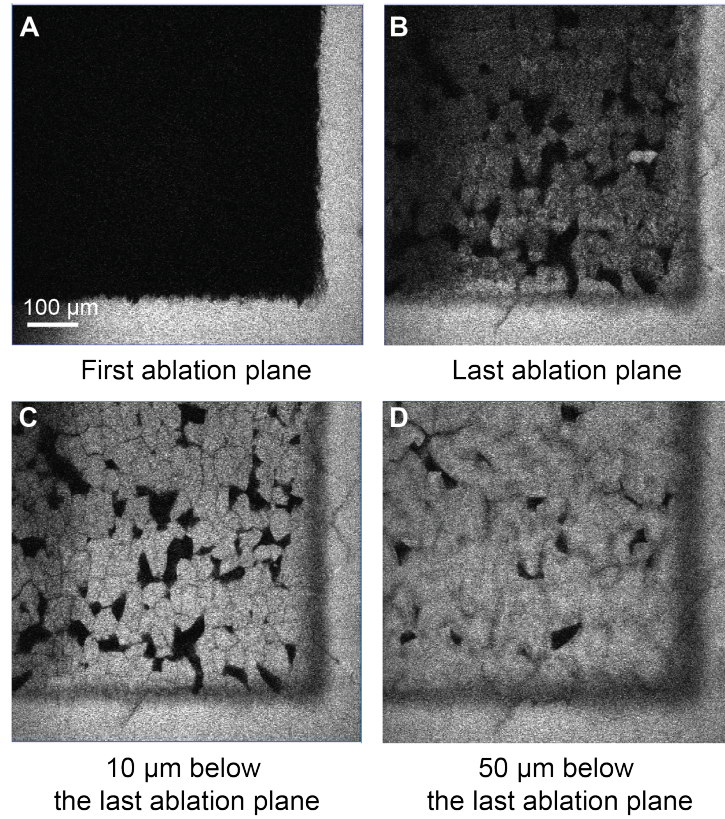


Figure 5.3. Continuous removal of fluorescent gelatin block using planar ablation pattern.

The fluorescent gelatin block was ablated continuously with the following parameters: fluence 7 J/cm^2 , fast axis speed 70 mm/s , slow axis step size $73 \mu\text{m}$, axial step size $10 \mu\text{m}$, ablation volume $700 \times 700 \times 50 \mu\text{m}^3$ (A) Image on the first ablation plane. (B) Image on the last ablation plane ($50 \mu\text{m}$ below the first plane). (C) $10 \mu\text{m}$ below the last ablation plane, the majority of the gelatin is preserved. (D) Plane ablation damages gelatin more than $50 \mu\text{m}$ below the last ablation plane.

should include the newly ablated surface, the intact imaged tissue, and the unimaged tissue. The optical penetrating length of the most scattering tissue determines the imaging depth, the ablation precision requirement, as well as the overall efficiency.

Serial imaging and ablation within the brain, housed within the skull, requires addressing tissue heterogeneity in both optical and mechanical properties. Different brain regions possess unique cellular compositions, leading to variations in scattering properties and mechanical strength between the gray and white matter. Outside the brain, the meninges are layered structures that comprise the dura mater, arachnoid membrane, and pia mater. The dura is tough and leather-like, the arachnoid is delicate and web-like, and the pia is thin and closely adheres to the surface of the brain. Moving outward, the thickness of the skull is inconsistent across its surface. Above the cortical region, the skull measures typically less than 100 μm , interspersed with a single layer of vasculature. Above the cerebellum, the skull's thickness can reach up to about 500 μm , housing multiple marrow cavities along the dorsal-ventral axis. Furthermore, the tissue within these skull cavities consists of hematopoietic and mesenchymal cells, which have notably lower mechanical strength than the neighboring rigid bone matrix, which is a dense composition of minerals like hydroxyapatite and collagen fibers. These multilayered bone and marrow structures introduce more pronounced optical distortion for deep tissue imaging compared to the cortex, while the proximity of these mechanically distinct tissues poses a challenge to precise laser ablation.

5.2.1 Establishing a compatible index matching protocol for laser ablation

For a fixed mouse brain in Phosphate-Buffered Saline (PBS), the scattering length l_s in the fixed brain tissue is about 100 μm , while the skull exhibits even stronger scattering ($l_s \approx 40\mu\text{m}$). As the calcified collagen fibers in the skull introduce strong scattering due to its high refractive index, we further decalcified the mouse head before index matching using 2% of EDTA solution.

We explored several commonly-used index matching protocols, paying special attention

to their compatibility with our ablation setup. Femtosecond laser ablation ionizes molecules and produces gas bubbles. The ionization process depends on the atomic and molecular structure of the molecule, while the gas bubble dynamics (e.g. merging, detaching from the surface, etc) depend on fluid viscosity and surface tension. 67% TDE solution is widely used for index matching[18], but its high viscosity prevents efficient removal of persistent bubbles. 40% OptiPrep 60% DMSO solution was used in our prior serial two-photon imaging for its relatively high refractive index and compatibility with fluorescent protein [131]. However, we found a significant amount of persistent bubbles generated more than 100 μm below the ablation plane under modest ablation fluence ($1\text{J}/\text{cm}^2$) at the focus. We postulated that this iodine-rich solution has a much lower ionization fluence threshold due to the abundance of outer shell electrons. After extensive testing, we selected 60% DMSO 40% water with 10 mM HEPES buffer solution (refractive index, RI 1.427) for its high index, low viscosity, and compatibility with fluorescence proteins[26]. For samples without fluorescence proteins, the concentration of DMSO could be increased to reach a higher refractive index, noticing the weak and nonlinear dependence of viscosity on the DMSO concentration in water[67].

For multi-day continuous acquisition, maintaining the concentration of the index matching solution is crucial. With an open surface between the sample holder and the objective, water evaporation and absorption are not negligible: the total volume of our flow system is about 1.2 L. Starting from 60% DMSO 40% H₂O solution, a 24-hour-long operation will increase the refractive index from 1.427 to 1.429. On the other hand, 90% DMSO 10% H₂O solution will absorb water from the atmosphere and therefore lower the solution index from 1.467 to 1.45 in a few days. The drift of the media refractive index changes the magnification of the objective and introduces extra optical aberrations. In addition, the corresponding changes in the solvent concentration also induce sample expansion or shrinkage, which further complicates both ablation volume detection and the subsequent image processing. Currently, we measure and adjust the refractive index every day so the index can be fixed at around 1.430 ± 0.001 .

5.2.2 STTP with a linear ablation pattern

To further confine the axial precision of the ablation, we replaced the spherical achromatic lens in the temporal focusing setup with a cylindrical lens (700 mm focal length, LJ1836L10B, Thorlabs, USA). This setup produces a line ablation pattern of size $107 \times 1 \text{ m}^2$ (Figure 5.4). This cylindrical lens focuses the diffracted beamlets from the grating without producing a line focus inside the objective, therefore eliminating potential damage to the objective lens. This modification is a compromise for the precision required for heterogeneous tissue ablation. For homogeneous material removal, the ablation area can be dramatically expanded to about $138 \times 32 \mu\text{m}^2$ by inserting a 0.25° diffuser (EDS-0.25, RPC Photonics, USA) right after the diffraction grating, which can be automated with a motorized filter flip (MFF101, Thorlabs, USA).

5.3 System hardware design

To image the complete vascular system in the skull and brain, we designed and constructed a system for automatic volumetric multiphoton imaging at sub-micrometer resolution. The system consists of a high-speed two-photon scanning microscope and a femtosecond laser ablation module.

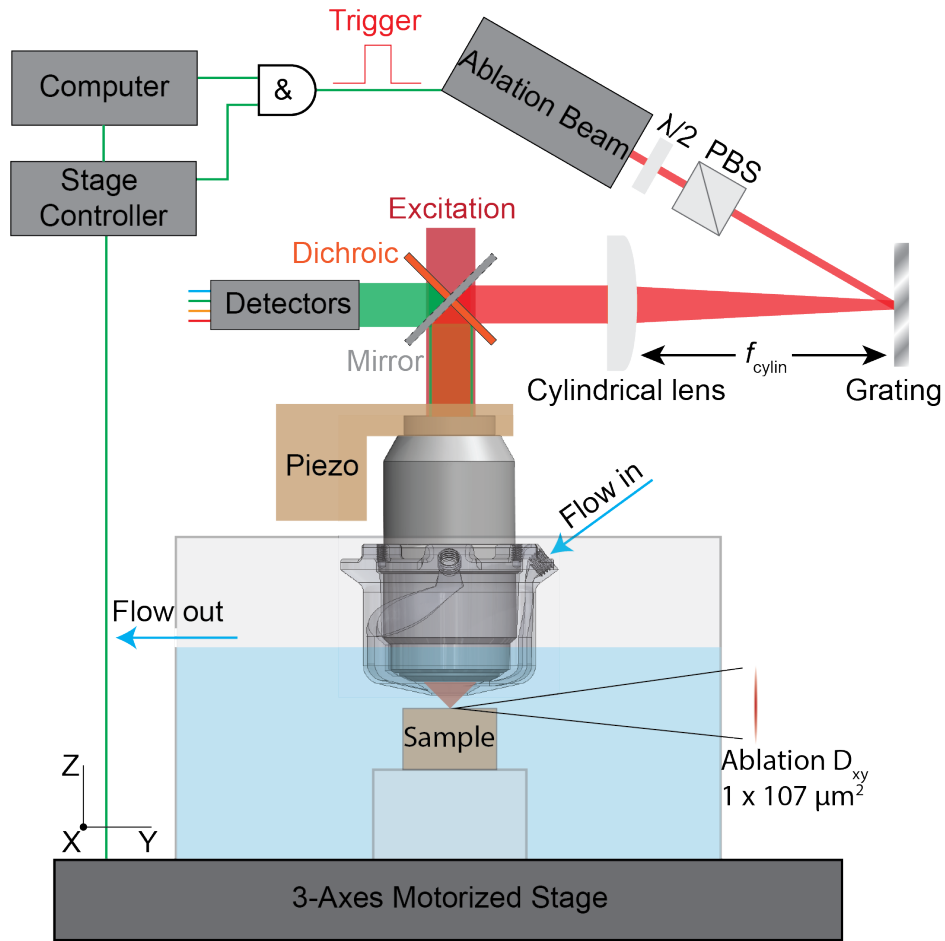


Figure 5.4. Microscope hardware diagram. The ablation beam passes through a half-wave plate and polarizing beam splitter(PBS) before incidenting on the grating. The surface of the grating is conjugated to the imaging focal plane through a telescope in 4f configuration, comprising a cylindrical lens and the high-NA objective. The ablation laser is triggered jointly by the computer and stage controller.

The imaging module consisted of a 4-channels two-photon microscope for high-resolution multiphoton imaging. The optical design was adapted from the Adaptive-optical two-photon microscope[72] described earlier for its optical performance. The detection part was revised to accommodate for 2-inch optics and 4 symmetric detection channels. Sourced by an ultrafast laser oscillator (Chameleon Discovery NX, Coherent USA), the beam is expanded, reflected by a Galvanometer scanner and an 8 kHz resonant scanner (Cambridge Technology, USA), further expanded to fill the back aperture of the objective (XLSLPLN25XGMP, 1.0 NA, 8 mm WD,

Olympus, Japan). A piezo stage (nPFocus400, NPoint., USA) was used for fast stack acquisition. Image acquisition was controlled by the ScanImage (MBF Bioscience, USA) software running in MATLAB (MathWorks, USA).

The imaging and ablation modules are combined right before the objective, by switching between the primary dichroic (FF775-Di01-36x50, Semrock, USA) and a mirror (PFR10-P01, Thorlabs, USA) using a motorized actuator (NA11B60-T4A, Zaber, Canada). To image and ablate samples on the scale of tens of millimeters at high resolution, high-precision mechanical stages are necessary to translate the sample (XY: LDA075A-AE53T10A, Z LRQ075AL-DE51CT10A, Controller: XMCC-4, Zaber, Canada).

5.4 System controlling pipeline

The microscope's operation is automatically controlled by custom software developed in MATLAB and Python. Users initiate the procedure by setting operational parameters, after which the microscope iteratively images and ablates the sample until task completion.

5.4.1 Imaging control

The microscope operates in two imaging modes: exploration and scan (Figure 5.5). In exploration mode, each captured tile is immediately analyzed; neighboring tiles exhibiting specific signal criteria are queued for acquisition. The exploration tiles also include volumes ablated previously. If analysis confirms a clean prior ablation surface, the microscope will transition to high-resolution imaging (Scan mode). Upon concluding the scan mode or if exploration reveals unsatisfactory prior ablation, the system activates the ablation mode.

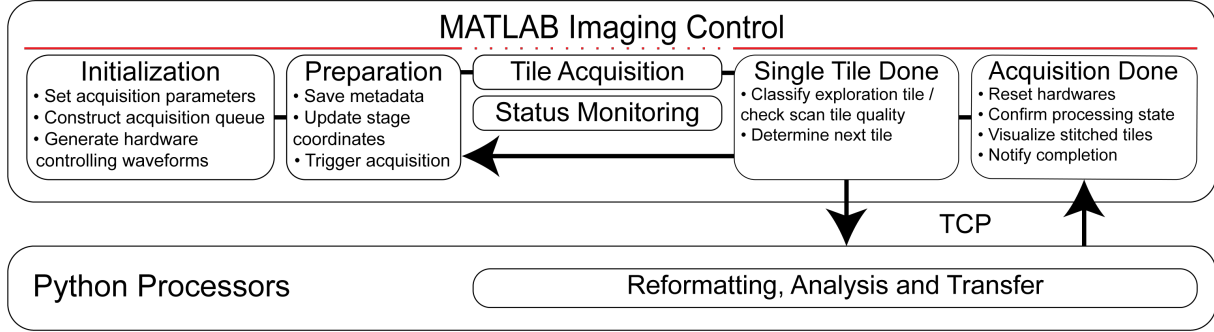


Figure 5.5. Microscope imaging flow diagram. The red line indicates the running time of the microscope control process. During tile acquisition, the control process periodically checks the hardware states.

In both imaging modes, the accessible space of the stages is discretized into smaller tiles based on acquisition parameters. Scan mode tile has $900 \times 1600 \times 200$ voxels at $0.35 \times 0.35 \times 1.0 \mu\text{m}^3$ voxel resolution, each one with $60 \times 100 \times 100$ voxel overlap with its 6 neighbors. Exploration mode tile has $700 \times 700 \times 61$ voxels at $1.0 \times 1.0 \times 5.0 \mu\text{m}^3$ voxel resolution without overlap. Benefiting from the built-in linear encoders on all stages, tile positions are defined using stage coordinates. This approach ensures ease of recovery from unanticipated operational disruptions and streamlines subsequent imaging stitching.

To guarantee long-term reliability, the status of the imaging laser is constantly monitored, with synchronous analysis of the acquired images for quality control. Communication between the controlling computer and the imaging laser was implemented using serial port. A MATLAB timer was implemented to query and log laser states, including laser power, wavelength, shutter state, and tuning state, at 1 Hz. In Scan mode, after the acquisition of each tile, we computed the maximum intensity projection of the acquired image along the z-direction. This projection was analyzed to detect potential de-synchronization between scanning and acquisition triggers. Detection of hardware malfunctions triggers a "re-image" notification to the controlling pipeline.

The acquisition of each image tile is controlled by ScanImage, which writes image data directly to a high-speed solid-state drive as a Tiff file. To overlap image processing with acquisition with minimal performance impact on the MATLAB-based control process,

we implemented an asynchronous parallel processing pipeline in Python. This pipeline de-interleaves the Tiff stacks and saves the image on high-capacity hard drives in HDF5 format to optimize input/output performance. Concurrently, a downsampled version of the image stack, along with relevant descriptive statistics, are saved separately for computing the ablation mask. The processing status is relayed back to MATLAB via the Transmission Control Protocol (TCP).

5.4.2 Ablation control

In the Ablation mode, the control software analyzes the imaged tiles to detect ablation volume and generates a hardware command sequence. This process commences with loading and stitching the downsampled imaged tiles at 5 μm isotropic resolution using the stage coordinates (Figure 5.7A). A linear spectrum unmixing algorithm is applied to reduce spectrum cross-talk between detection channels. Subsequently, intensity-based channel-specific image segmentation algorithms determine the ablation volumes. The results are analyzed to identify ablation targets, including the skull, tissues in the skull cavities, and brain tissues, which are summarized in a 3D labeled array (Figure 5.7B).

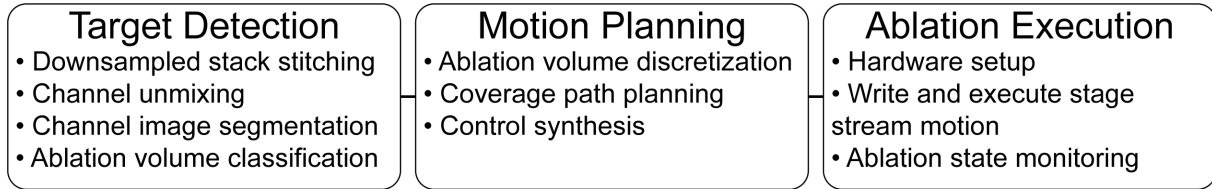


Figure 5.6. Microscope ablation flow diagram.

Both the labeled array and sets of ablation parameters specify the command sequence. For each ablation target, ablation parameters include fast axis speed, slow axis step size, effective peak ablation fluence, and z-positions. The fast axis corresponds to the top linear stage which can move most rapidly with the least load. For at a given z-position, a 2D mask of an ablation target at 1 μm resolution is computed from the labeled array via nearest neighbor interpolation. This 2D mask is decomposed into parallel stripes along the fast axis (X-axis in Figure 5.4). The widths of these stripes equal the slow axis step size, which is the product of the width of the ablation

pulse along the slow axis and the overlapping fraction. For each stripe, the y-intervals containing the ablation target are identified. The y-position of the ablation target in the stripe defines the position for the change of triggering signal, while an additional acceleration/deceleration distance is added to both ends to ensure the motorized stages can reach a constant speed when the ablation laser is triggered. Lastly, we search for an efficient route that traverses all intervals as a coverage path planning problem. Intervals are clustered into regions based on their spatial proximity and overlapping fractions. These regions form the nodes in the Travel Salesman Problem (TSP), where the distance between two nodes is the minimal distance between any endpoints of these two regions. To specify the starting node and remove the requirement of returning to the starting point in the TSP problem, a dummy node with 0 distance from all the nodes, 0 distance to the specific starting node, and infinity distance to all the other nodes is introduced. The approximated shortest path traversing all regions is found using the 2-opt algorithm (Figure 5.7C). For 3D ablation, the end position of the ablation path in the previous plane was used to initialize the path planning in the current plane (Figure 5.7D).

Finally, the path, triggering positions, and kinematic parameters are written to the stage controller stream buffer and executed. As the stage controller uses the idealized motion for trigger output, an additional offset is introduced to compensate for the delay between actual movement and the control input. This offset distance is empirically determined as the product of the typical delayed time and the stage linear motion target speed.

The ablation process is specified by the following parameters:

- γ_f : numerical scalar, effective normalized ablation spot size along the fast axis. The translation speed of the stage $v_{abl} = \gamma d_f f_L$, where d_f is the diameter of the ablation spot along the fast axis, f_L is the repetition rate of the ablation laser.
- γ_s : numerical scalar, effective normalized ablation spot size along the slow axis. The spacing between the ablation stripes $\Delta y = \gamma_s d_s$, where d_s is the diameter of the ablation spot along the slow axis.

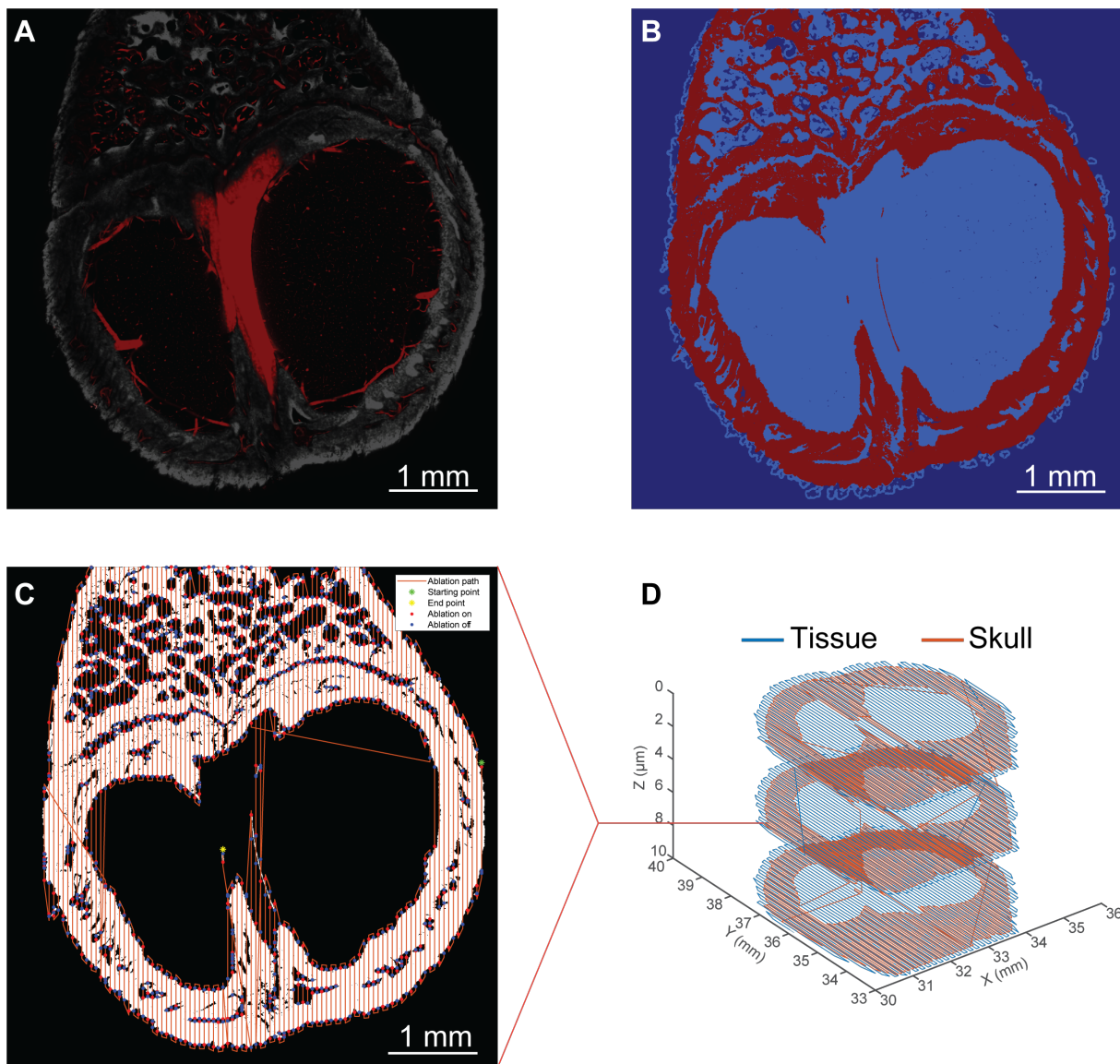


Figure 5.7. Ablation path generation. (A) Dual channel image of brain vasculature (red) in skull (gray). (B) Dual channel image segmentation of tissue (light blue), skull (red), and background (dark blue). (C) Ablation path of the skull on one ablation plane. (D) Ablation path of skull and tissue in 3 consecutive ablation planes.

- Δz : numerical scalar, the spacing between adjacent ablation sections.
- F : numerical scalar, estimated ablation fluence (J/cm^2).

In the following text, we report ablation parameters in the following order: $(\gamma_f, \gamma_s, \Delta z, F)$.

Hydrodynamic constraint on plane ablation efficiency

In large-scale tissue ablation, motorized stages move the sample across the objective's focal point, potentially in a back-and-forth manner. The transnational speed is the product of the effective pulse size in the fast translating direction and the laser repetition rate. Enhancing ablation efficiency can be achieved by expanding the ablation laser beam incident on the grading and utilizing a laser with a higher repetition rate. However, the liquid in the sample holder introduces hydrodynamic limits on the translation speed, thus limiting the overall efficiency.

Consider a rectangular container of length L , partially filled with water of height h , oscillating horizontally with a cyclic frequency Ω . In the moving coordinate, the deviation $\zeta(x, t)$ of the liquid surface from its equilibrium position, due to container oscillation, is given by[12]:

$$\zeta(x, t) = h - \frac{\Omega U_0}{g} \left\{ \left(\frac{1}{2}L - x \right) + \frac{4L\xi}{\pi^2} \sum_{n=1}^{\infty} ' \frac{\cos(\pi x n/L)}{n^2(n \tanh(nr) - \xi)} \right\} \sin \Omega t, \quad (5.1)$$

where U_0 is the maximum velocity of the oscillating coordinate, g is the gravitational acceleration, $\eta = L\Omega^2/\pi g$, $r = \pi h/L$, and the prime indicates summation over odd integers only. Consequently, the resonant frequencies are given by:

$$\Omega = \sqrt{\frac{n\pi}{L} g \tanh\left(\frac{n\pi}{L} h\right)}. \quad (5.2)$$

For our sample holder around 11.4 cm, with a liquid level h of approximately 5 cm, the first resonant frequency is about 15.5 Hz. This frequency gives the lower limits on the acceleration duration $t_{\min} = \Omega^{-1}$. To avoid resonance, we set the minimal acceleration duration to 86 ms.

5.5 Results

Currently, the system is able to iterate between imaging and ablation to capture the 3D structure of brain vasculature within the skull. Starting from the top of the brain mounted horizontally, we imaged FITC-labeled vasculature and skull at $0.35 \times 0.35 \times 1.0 \mu m^3$ resolution using two-photon excitation and second harmonic generation. After imaging a $150 \mu m$ horizontal section, $75 \mu m$ of brain tissue and skull were removed. For the top $40 \mu m$, tissue and skull were removed at $(2, 0.75, 10 \mu m, 6 J/cm^2)$ and $(1, 0.5, 5 \mu m, 10 J/cm^2)$, respectively. For the remaining $35 \mu m$, tissue and skull were removed at $(2, 0.75, 8 \mu m, 5 J/cm^2)$ and $(1, 0.5, 3 \mu m, 6 J/cm^2)$, respectively. After 5 iterations, the microscope removed the skulls above the sagittal sinus and part of the transverse sinuses (Figure 5.8) while maintaining a relatively stable optical resolution for vasculatures in both brain parenchyma and skull cavities.

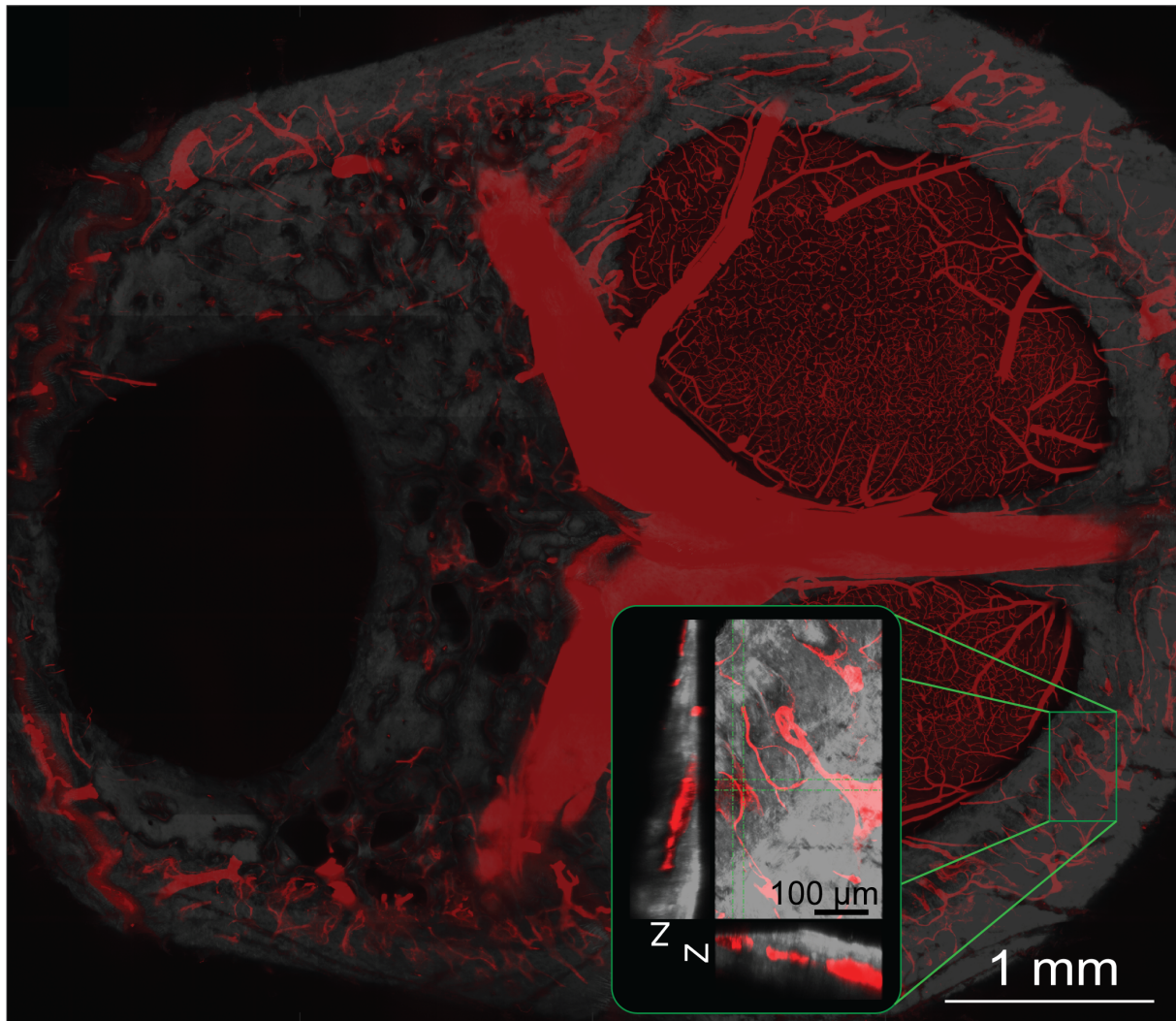


Figure 5.8. 150 μm max intensity project of mouse brain vasculature inside the skull. Vessels are filled with FITC gelatin (red). The skull (gray) is imaged using Second Harmonic Generation (SHG). The inserts show the high-resolution image along with projections along the two lateral directions. The scale bar for the insert is 100 μm

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