Development of a Novel Imaging Methodology for Quantitative Analysis of the Mouse Cortical Vasculature

by

Joe Steinman

A thesis submitted in conformity with the requirements
For the degree of Master of Science
Graduate Department of Medical Biophysics
University of Toronto

©Copyright by Joe Steinman 2013
Development of a Novel Imaging Methodology for Quantitative Analysis of the Mouse Cortical Vasculature

Joe Steinman
Graduate Department of Medical Biophysics
University of Toronto

Abstract

This thesis describes a novel imaging methodology for visualization and quantitative analysis of the vascular topology of different cortical regions of the mouse brain in 3D. The brain is perfused with a fluorescent contrast agent, and rendered fully transparent via optical clearing. This procedure enables images through the whole cortical depth to be obtained with a 2-photon microscope without sectioning. The Allen Reference Atlas (ARA) (Lein et al., 2007) is registered to the 2-photon data for delineation of the cortical regions. Quantitative metrics are then extracted from the different regions using an automatic vessel segmentation algorithm. These metrics are compared with those obtained by other investigators to validate this technique, and are found to be in agreement. Since this methodology possesses the resolution to visualize vessels of all sizes, and provides reasonable estimates of quantitative parameters, it shows strong potential for quantitative analysis of normal and abnormal cortical vascular architecture.
Acknowledgements

I would like to thank my supervisor Dr. John G. Sled for his support. I enjoyed our many fruitful discussions, and appreciate his helpful advice, guidance, and time and care shown me. His approach to problem solving was patient and creative. The computer skills he taught me were numerous and complex. The learning experience and opportunity he provided were unmatched. He played an important role in making the MSc a success. I can’t thank him enough.

I would like to acknowledge Dr. Bojana Stefanovic (Committee Member and Collaborator) for her valuable comments and suggestions on developing the imaging methodology, data analysis, and additional metrics for extraction from the data. I thank her for always and promptly responding to my numerous emails and answering my questions. I express my sincere appreciations to Dr. Mark Henkelman and Dr. Bob Harrison for their encouragement and recommendations as my Committee Members on my thesis and presentations.

I appreciate the assistance of Adrienne Dorr in developing the data acquisition protocol. I value the hours spent with me at the microscope, and for her training in the usage of the 2-photon microscope. Her efforts in showing me the correct usage of image visualization programs such as Imaris, and finding online the stitching program used in this thesis (XuvTools) were considerable.

I would like to thank Dr. Lindsay Cahill for patiently answering my questions, and for passing to me many of the valuable skills she acquired at MICe. I would like to thank Dr.Lisa Yu and Shoshana Spring for their advice on perfusions during the project’s outset.
Colin Gram and I had many useful discussions, while Christine LaLiberte assisted me with various laboratory techniques. I thank Matthijs van Eede for his creativity and advice in solving various computer and programming related complications.

I thank my parents for their support and encouragement.
Table of Contents

Abstract.................................................................................................................................................. ii

List of Tables......................................................................................................................................... vii

List of Figures......................................................................................................................................... viii

Chapter 1 ................................................................................................................................................ 1

1.1 Introduction...................................................................................................................................... 1

1.2 Structure and Organization of the Thesis.......................................................................................... 2

1.3 Rationale for the Development of an Imaging Methodology for Visualization of the Cortical Vasculature ....................................................................................................................... 2

  1.3.1 Biological Significance of the Cerebral Vasculature................................................................. 3

  1.3.2 Vascular Variations Across Cortical Regions ........................................................................... 5

  1.3.3 The Need to Develop a Novel Imaging Methodology............................................................... 7

1.4 Cellular and Vascular Biology of the Cortex .................................................................................. 10

  1.4.1 The Cerebral Cortex................................................................................................................. 10

  1.4.2 Cerebral Cortical Vasculature................................................................................................. 14

  1.4.3 Cortical Parcellation Schemes ................................................................................................. 16

  1.4.4 Mouse as a Model of Human Neocortex................................................................................ 17

1.5 Physics and Technology.................................................................................................................. 18

  1.5.1 Light Interaction with Tissue................................................................................................... 18

  1.5.2 2-Photon Microscopy.............................................................................................................. 19

  1.5.3 Optimization for Deep Tissue Imaging.................................................................................... 22

  1.5.4 Aberrations in Microscopy...................................................................................................... 25

  1.5.5 Vessel Segmentation............................................................................................................... 28

1.6 Summary of Introductory Chapter.................................................................................................... 29

Chapter 2 ................................................................................................................................................ 30

2.1 Justification for a New Imaging Methodology for Visualization of the Mouse Cortical Vasculature ........................................................................................................................................ 30

2.2 Specimen Preparation Protocols and Imaging ............................................................................. 34
2.2.1 Perfusion of the Vasculature with a Fluorescent Contrast Agent ...........................................34
2.2.2 Fixation, Dehydration, and Optical Clearing ........................................................................36
2.2.3 2-Photon Microscopy and Data Acquisition ........................................................................36
2.2.4 Delineation of the Cortical Regions in the 2-Photon Data ......................................................41

2.3 Establishing the Tools and Analysis Methodologies for Evaluating Vascular Topology ........42
2.3.1 Vessel Tracking ......................................................................................................................42
2.3.2 Processing of Vessel Tracking ...............................................................................................44
2.3.3 Extracting Penetrating Vessels and Defining the Capillaries ..............................................49
2.3.4 Calculating Depth Below the Cortical Surface of a Vessel Segment ..................................50
2.3.5 Extracting Quantitative Parameters .....................................................................................53

2.4 Summary of the Results Obtained with the Imaging Methodology ........................................55

Chapter 3 ........................................................................................................................................ 57

3.1 Advantages of the Imaging Methodology ..................................................................................57
3.2 Limitations of the Imaging Methodology ..................................................................................63
3.3 Future Research and Applications of the Technique .................................................................67

References ........................................................................................................................................ 70
List of Tables

Table 2.1 Morphometric parameters according to cortical region .......................................................... 55
List of Figures

Figure 1.1 Laminar organization of the cerebral cortex ................................................................. 13

Figure 2.1 (A) Maximum Intensity Projection of the 2-photon data set analyzed in this thesis (B) Seed placement based on local intensity maxima .................................................................................. 40

Figure 2.2 Isosurface rendering of the segmented vasculature .......................................................... 47

Figure 2.3 Location of 2-photon data relative to the whole brain ...................................................... 48

Figure 2.4 (A) Centerlines of all penetrating vessels extracted from the segmented vessel network (B) Separation of capillaries from non-capillaries based on penetrating vessel extracting and diameter cutoff .......................................................................................................................... 50

Figure 2.5 (A) Defining the cortical surface (B) Magnified view of (A) ............................................... 52

Figure 2.6 Plot of capillary length density vs cortical depth ............................................................ 54
Chapter 1

1.1 Introduction

This thesis focuses on the development of a methodology for imaging large portions of the mouse cerebral cortical microvasculature in three dimensions. Quantitative metrics on the vasculature are extracted and compared to assess the validity of the technique. Ultimately the methodology will be used to compare the vascular architectures of different cortical regions, which will shed light on how the architecture enables tissue metabolic demands to be met, homeostasis to be maintained, and metabolic waste products to be removed from tissue. This thesis establishes the techniques and analytical tools for making this comparison.

The goals of this introductory chapter are: (1) To provide a rationale for the development of a new imaging methodology (2) To explain the rationale for the application and usage of this new methodology to studying the vasculature of different cortical regions and (3) To provide background information to better understand the research described in the thesis.

The introduction is divided into three body sections. The first outlines the motivation for the thesis: the biological significance of the vasculature, the benefits of understanding how the vasculature varies across the brain, and how development of a new imaging technique will provide insight into the organization of the vasculature. The need and role of a new imaging methodology will be covered. The next section supplies the biological background: general structure and organization of the neocortex and its vasculature, cortical parcellation schemes, and use of the mouse as a model of the human neocortex. Its purpose is to improve understanding and familiarity with the brain regions and its vasculature referred to in the study. The final section is devoted to the relevant physics and technology. This includes light interaction with tissue, 2-photon microscopy, optimization of 2-photon for deep tissue
imaging, and aberrations in microscopy. This section details some of the rationale for the specimen preparation and imaging protocols detailed in Chapter 2, and outlines the structure and organization of the thesis.

### 1.2 Structure and Organization of the Thesis

Chapter 1 provides the rationale and motivation for the comparison of vasculature across the brain, and outlines the general characteristics and purpose of the cerebral vasculature and cortex.

The succeeding section describes how 2-photon microscopy, combined with optical clearing, was used in this project to image large portions of the cortical vasculature. A method for delineating cortical regions in the 2-photon data is advanced, and automatic vessel segmentation techniques are employed to extract quantitative parameters from the 2-photon fluorescence microscopy images.

The thesis continues and concludes with a discussion and analysis of the results. Limitations and future applications of the imaging methodology are considered.

### 1.3 Rationale for Development of an Imaging Methodology for Visualization of the Cortical Vasculature

Three general motivations were considered in preparing the thesis: (1) the benefits to studying the cerebral vasculature (2) the importance of comparing cortical regions and (3) the importance and relevance of advancing an imaging methodology to study these regions’ vasculature. These motivations are described below.
1.3.1 Biological Significance of the Cerebral Vasculature

The purpose of the cerebral vasculature is the transport of materials such as oxygen, glucose, and other nutrients (vitamins, minerals, amino acids, and fatty acids) throughout the brain. It is further responsible for removal of metabolic waste. A specialized vascular architecture has been developed in the mammalian brain to regulate and maintain the distribution of blood (Nishimura et al., 2007). Large cerebral arteries supply blood to the surface of the cortex through an interconnected network of vessels consisting of a grid of abundant loops (Blinder et al., 2010). These vessels are termed pial vessels. Branching from them are the penetrating arterioles/venules that enter the tissue perpendicular to the cortical surface and traverse the cortical depth. Branching from the penetrating arterioles is a capillary network with a net-like structure. Capillaries are thin (typically less than 10 µm in diameter), and it is from inside the capillaries that oxygen, glucose, and nutrients diffuse out of the vascular network and into the tissue, and metabolic waste products such as carbon dioxide diffuse from the tissue into the vasculature. Capillaries intersect with the venular network, and the penetrating venules transport de-oxygenated blood to the cortical surface.

This organized network enables the efficient delivery of oxygen and glucose to neurons, which is essential since neurons do not naturally store or produce these molecules. Oxygen and glucose are necessary since they are used by neurons and neuroglia to produce Adenosine Triphosphate (ATP), which stores energy needed by these cells to carry out their functions. Neurons require ATP for protein synthesis and axoplasmic transport, neurotransmitter packaging for cell-to-cell communication, and operation of ion pumps to restore unequal charge distributions following action potentials (Huettel et al., 2004).

Increased neuronal activity is supported by increased Cerebral Blood Flow (CBF) for increased oxygen and glucose delivery to neurons. CBF is dependent upon a variety of factors such as blood pressure; the
diameter of the blood vessel; the density of the red blood cells; and the angioarchitecture, which is the arrangement and patterning of blood vessels (Huettel et al., 2004). As the total resistance of a vascular network to flow is related to the angioarchitecture, blood flow and hence oxygen delivery is constrained by this architecture, and impacts potential neuronal activity in those regions.

Abnormal vasculature may be present in disease. Cerebral capillary degeneration, and increased vessel tortuosity, are common in Alzheimer’s Disease (de la Torre, 2002; Brown et al., 2002). Cortical microinfarcts have been shown to affect cognition in brain aging (Kovari et al., 2004). To determine if these changes are important in disease, it is important to obtain information on cortical vascular topology together, which together with blood flow modeling may improve understanding on how these disruptions in the network structure affect blood flow. Analysis of the microvessel architecture in healthy brains will uncover topological properties of normal vascular trees, and may aid in identification of disease states where the vasculature differs in a functionally different way from the healthy state. An additional motivation to study the cortical vasculature is to understand the importance of genetic mutations and factors in their contributing to disease. Genetic mutations are known to affect vascular development and may predispose humans to stroke (Proweller et al., 2007); studying their effect on the vasculature could clarify their role in stroke and other vascular-related disorders.

Understanding the microvasculature is useful for research in hemodynamic based functional imaging, specifically the Blood Oxygenation Level Dependent signal (BOLD) in functional Magnetic Resonance Imaging (fMRI). The BOLD signal arises when neuronal activity triggers a hemodynamic response via neurovascular coupling. The vascular basis of the BOLD signal is an imbalance between increases in cerebral blood flow (CBF) and increases in oxygen metabolism (Arthurs and Boniface, 2002). While the epicenter of blood flow response is in the region of neural activity, it has been noted that the hemodynamic response occurs over a region larger than that where neural activity has increased
This suggests that the distribution of hemodynamic responses measured with fMRI may be constrained by the architecture of the microvascular blood supply. In other words, the architecture could limit the resolution of activation maps.

A database of microvessel geometrical properties may shed light on the relationship between brain activity and vascular signals, leading to more accurate interpretations of the BOLD signal. The BOLD signal is only an indirect measure of neuronal activity, and the signal recorded, though correlated with neuronal activity, is related to the magnitude of the hemodynamic response (Thomason et al., 2005). The precise quantitative relationship between neuronal activity and the BOLD signal is not yet known, yet the relative contributions between the vascular and metabolic components of the response must be known to accurately quantify neuronal activity. Regional differences in BOLD reactivity between resting and activated neuronal states have been found in primary and association cortex (Ances et al., 2008). Together with observed regional differences in baseline CBF, and regional CBF differences when hemodynamic responses independent of neural activity are induced (such as hypercapnia) (Lorthois et al., 2011), these results suggest that the microvascular anatomy may influence the BOLD signal. Further, due to different vascular densities and (possibly) architectures in different cortical areas, direct comparison of fMRI results between areas may be inappropriate (Lorthois et al., 2011; Harrison et al., 2002). Such comparisons may be more accurately made with a database of regional microvessel geometrical properties, which should help in separating the vascular and neuronal contributions in the BOLD signal.

1.3.2 Vascular Variations Across Cortical Regions

The cerebral cortex is a thin layer of tissue covering the pair of cerebral hemispheres. It is responsible for diverse functions such as movement, speech, vision, thought, hearing, and emotion. It is implicated in disease. For example, in Alzheimer's, as the disease spreads through the cortex via deposition of
plaques and tangles (Arnold et al., 1991), there is a decline in judgement and language is impaired. Studying the cortex may provide insight into its structure and functionality, and clarify its involvement in neural diseases.

Cortical regions differ in metabolic activity, density, thickness of cortical layers, neuronal connectivity, and cell types. Wree et al. (1990) established that when the laminar distribution patterns of local cerebral glucose metabolism are compared between cortical regions, the area borders correspond almost exactly with the cytoarchitectonically defined borders in Nissl-stained preparations. The confirmation by Borowski and Collins (1989) of a positive correlation between capillary density, and glucose utilization rate, suggests that microvessel density is potentially one way to distinguish between cortical regions. This idea is supported in the literature: Zheng et al. (1990) and Keller et al. (2011) found that the total number and length density of microvessels is greater in highly active functional modules in the primate visual cortex.

Some studies suggest that distribution of nerve fibres (i.e. neuronal connectivity) may influence vessel geometry. Snodderly et al. (1992) described a parallel orientation of capillaries to the nerve fibres in the primate retina, and Lewis et al. (1987) found patterns of regional and laminar specialization of fibers in primate neocortex. This suggests differences in neuronal connectivity between regions may lead to differences in capillary geometry or orientation. Additionally, secretion of Vascular Endothelial Growth Factor (VEGF) during brain development by Schwann cells induces arteries to grow alongside the nerve fibres (Carmeliet, 2003), suggesting a relationship between vessel and fibre orientation. With respect to neuronal density, Tsai et al. (2009) showed a positive correlation between neuronal and vessel density on a regional level. Their results indicated a trend for neuronal and microvascular densities to decrease as one moves laterally along the cortical mantle from entorhinal to cingulate cortex.
Each region possesses a unique cellular architecture, and it is unknown whether the vasculature is organized in patterns corresponding to these structural features. Vascular patterning differences may provide insight into the organization of the cortex (Zheng et al., 1990), and shed light on how networks are arranged to meet a tissue’s metabolic demands. Presently, a comprehensive comparison of vascular architectures between regions has yet to be conducted. This thesis aims to establish and validate the tools and techniques for making this comparison possible.

1.3.3 The Need to Develop a Novel Imaging Methodology

Importance of 3D imaging in vascular research

Studies of the vasculature have traditionally relied on imaging techniques such as corrosion casting and histology. In corrosion casting, the vessels are filled with a plastic resin, the tissue is corroded, and the vascular cast is imaged with a scanning electron microscope, or with optical techniques for visualization of large vessel architecture. Vascular networks are geometrically complex, three-dimensional (3D) networks, and 3D imaging methods are required to capture this complexity. The scanning electron microscope images are difficult to analyze in 3D: they are a 2D projection of a 3D structure, similar to taking a photograph with a camera. As a result, accurate quantitative metrics are difficult to obtain with this method. For example, Minnich et al. (2000) found vessel lengths measured from electron microscope images to be underestimated by an average of about 12% since depth information is not contained in the images.

With histology, the vasculature is filled with an opaque material such as India Ink, and the brain subsequently sectioned for visualization with light microscopy. Examples of quantitative parameters that may be extracted are vascular volume, length, surface area, density, and vessel diameters (Weber et al., 2008). However, tissue slicing introduces distortions that impact network topology, or affect quantitative accuracy. For example, slices may be torn during processing. Branch points are lost where
the tissue is sliced. Further, the mechanical stress induced by the microtome bends and twists the tissue section. The bending angle has been estimated in some cases to be around 10°. Large vessels also fall off the sections during processing (Weber et al., 2008). These large vessels are scarce; however their contribution to the vascular volume is significant. Furthermore, histology-based studies show limited imaging depth due to the penetration ability of the illuminating light (Hirsch et al., 2012), which is at most a few hundred micrometers, or less with standard microscopy. Quantification of true volumetric parameters is often difficult to achieve. O’Flynn et al. (2007) found bifurcation angles of arteries measured with 2D methods to be up to 10° different from 3D measurements; on average vessel lengths were underestimated by about 8%.

3D imaging and analysis techniques to characterize vessel networks overcome the deficiencies in histology. They have been used in mice (Heinzer et al., 2006; Moy et al., 2013), humans (Cassot et al. 2006, 2009, 2010; Lauwers et al., 2008) and non-human primates (Risser et al., 2009). Typical imaging modalities that have been employed include µCT, confocal microscopy, and 2-photon fluorescence microscopy due to the high resolutions attainable. When vessel segmentation techniques are combined with the imaging, the topology of both non-capillaries and capillaries can be extracted.

The 3D data may further be applied to investigate blood flow through fluid dynamic modeling. For example, Lorthois et al. (2011) simulated blood flow in the cortical circulation to predict blood pressure, flow, and hematocrit distributions. These simulations elucidate the connection between the structure of the network (topology) and its function (blood flow and nutrient delivery to tissue). The topology influences the distribution of red blood cells (RBCs), and the total resistance of the network is dependent upon how each individual vessel is connected to one another (Hirsch et al., 2012). Each vessel has its own resistance to fluid flow based on its geometry, which is modified by the presence of RBCs. The resistance of an individual vessel is proportional to its length and the viscosity of the plasma,
and inversely proportional to the fluid density and its diameter (to the 4\textsuperscript{th} power) (Hirsch et al., 2012).

Network topology also affects RBC behavior at bifurcations (Hirsch et al., 2012). For example, RBCs tend to migrate towards a vessel midline, resulting in a region with relatively few cells near the vessel walls. As a result, plasma flowing into small side branches contains relatively fewer RBCs. This is compounded by the requirement of RBCs to overcome a deformation energy barrier in order to squeeze into a small blood vessel (Hirsch et al., 2012). Flow analyses have been performed on 3D data sets, with typical volumes ranging from 1.6 mm\textsuperscript{3} in human cerebral cortex (Lorthois et al., 2011) down to 0.003 mm\textsuperscript{3} in mice (Secomb et al., 2000). Generally, smaller field of view data sets are more difficult to perform flow simulations on as proportionally more vessels in the volume enter and exit the field of view, or are directly connected to another vessel that leaves the field of view.

\textit{Role for a new imaging method}

Ideally a methodology for cortical region analysis will provide resolution for visualization of all blood vessels. It will enable imaging large portions of intact cortex not distorted by sectioning, combined with vessel segmentation techniques for quantitative analysis. Imaging of large portions of cortex is important as significant vascular variations have been shown to exist within a cortical region (Keller et al., 2011), and large volumes of data are required to capture these variations.

A number of imaging methodologies have been developed and described. They are unsuitable for analysis of large cortical regions in a mouse brain, as they typically require the tissue to be sectioned to image deep into the cortex. For example, Moy et al. (2013) cleared mouse brain tissue in FocusClear (CelExplorer Labs, Taiwan), a dimethyl sulfoxide based optical clearing agent, and achieved imaging depths up to 850 µm. Since the cortical thickness can occasionally extend to 1.5 mm in mice (Lerch et al., 2008), to ensure imaging through the cortical depth, the brain was sectioned into 1 mm thick coronal slabs. Images covering the entire section, including the cortex and white matter, were obtained by Moy.
et al. (2013) and stitched together. This methodology is limited to 1 mm thick slabs of tissue, and information on vessel connectivity is lost at the cutting boundary as the sectioning procedure disconnects vessels from one another. Similarly, confocal microscopy on opaque human brain tissue forced Lauwers et al. (2008) to section their brain into 300 µm thick slabs due to the limited depth penetration of the confocal microscope. Neither of these techniques are suitable for imaging large areas of unsectioned cortex in three dimensions.

1.4 Cellular and Vascular Biology of the Cortex

This section describes the cerebral cortex, focusing on its function, cellular characteristics, laminar organization, and neuronal connectivity. These features vary across the brain and are the source for vascular differences. A general description of the cerebral vasculature is then provided, based on Duvernoy (1981). The patterns described by Duvernoy are found throughout the cortex, and provide a basis for understanding the cortex’s vascular architecture. Cortical parcellation schemes and their relevancy are discussed, since they highlight additional differences between regions. The accuracy of the mouse as a model of human cortex is examined. While generally the mouse cortex possesses many similarities to the human case, differences exist that must be considered when extrapolating data from mice to humans.

1.4.1 The Cerebral Cortex

The cerebral cortex is a layer of tissue at the edge of the cerebrum in the mammalian brain containing neuronal cell bodies and their unmyelinated fibres. It is responsible for functions that underlie perception and cognition. Cortical regions with simpler functions are termed primary cortices. An example of a simpler function is recognizing contours or edges, whereas a more complex function involves recognition of an object (i.e. a toaster). Primary cortices may receive direct sensory input
(somatic sensation, vision, hearing) and be directly involved in producing eye or limb movements. The association cortices subserve complex functions, and are adjacent to the primary cortices. They are involved in movement synthesis, attention, judgement, emotion, memory, language, creativity, and abstraction.

The properties of groups of cortical neurons determine how the brain functions. There are 2 types of cortical neurons: excitatory (pyramidal) and inhibitory. The term pyramidal derives from the presence of a large apical dendrite that points superficially. A neuron belongs to the layer in which its cell body is situated, even if its apical and basal dendrites span several layers. Pyramidal neurons are cloaked in spines, which are protrusions from dendrites that receive input from the synapse of an axon. The number of spines in a given layer indicates the likelihood of a neuron to sample signals in that layer.

Inhibitory neurons comprise only 20% of all cells, however they possess a more diverse morphology than their excitatory counterparts. For example, the axons of basket cells wrap around cell bodies in a ‘basket’-like shape; for chandelier cells, branching axons make multiple strings of contact (‘chandeliers’) around initial axon segments (Shipp, 2007). Inhibitory neurons are termed “interneurons” since their axons are intrinsic and they do not enter white matter, making only local short range connections. Most interneurons use the inhibitory neurotransmitters GABA (gamma-Aminobutyric acid) or glycine, though a few are excitatory.

The neocortex generally has 6 horizontal cellular layers, though some cortical areas, termed agranular cortex, largely lack a 4th cellular layer. An example of such a region is the motor cortex. The neurons in the various layers vertically connect to form columns. These layers are responsible for some of the vascular variations within a cortical region, as they each possess different metabolic activity levels. For example, in Saimiri primary visual cortex, there is a maximum difference in cytochrome oxidase activity (which is proportional to metabolic activity) of 64% between layer 1 and the upper subdivision of layer
4c, and 54% in *Macaca* between layer 5 and the upper subdivision of layer 4c (Keller et al., 2011). In rodent primary somatosensory cortex, metabolic activity (as measured with 2 deoxyglucose autoradiography) is 17% higher in the 4\textsuperscript{th} cortical layer compared to the first, and 40% higher in the 4\textsuperscript{th} layer compared to the 6\textsuperscript{th} (Hammer and Herkenham, 1983). Variations in vascularity through the cortical depth have been demonstrated in rodent (Masamoto et al., 2003), human (Lauwers et al., 2008), and monkey (Weber et al., 2008; Keller et al., 2011) cortex.

Information flow and processing in the brain depends on how these layers are connected, and the properties of the cells within each layer. Layer 1 is immediately beneath the pia mater and contains few cell bodies; it is termed the ‘molecular layer’. Layer 2 consists of small, densely packed cell bodies, which are largely pyramidal and stellate neurons, and is termed the ‘external granular layer’. Layer 3 is thicker than the preceding layers, and the pyramidal cell somata increase in size from superficial to deep locations. This layer is the ‘external pyramidal layer’. Layer 4, the ‘internal granular layer’, is densely packed with somata of small pyramidal and non-pyramidal cells. It is the main target of thalamocortical afferents from thalamus neurons, and intra-hemispheric corticocortical afferents. Layer 5, the ‘internal pyramidal layer’, contains large pyramidal cells. The ‘multiform layer’, or Layer 6, consists of densely packed, small cell somata which are modified pyramidal cells (Jones 2004).

It is possible to stain for axons and dendrites to visualize neuronal connections (see Figure 1.1). The direction in which fibre bundles are oriented enable the layers of the cortex to be visualized with this method.
Figure 1.1 Laminar organization of the cerebral cortex as depicted with three types of stains.

Different cortical regions may be distinguished by variations in layer thickness, cell type, cell density, and neurochemical markers. Each region has unique patterns seen in Nissl and myelin-stained preparations. The identification of different regions based on these stains is referred to as cytoarchitectonics or myeloarchitectonics depending on usage of Nissl or myelin stains.

Each layer possesses different cortical and sub-cortical connections that impact how data flows and is interpreted in the brain. Layers 1-3 are the target of interhemispheric corticocortical afferents, and the 3rd layer is the main source of corticocortical efferents. The pyramidal cells in the 2nd and 3rd layers provide the majority of corpus callosum axons. Most thalamic fibers terminate in the 3rd and 4th layers. Pyramidal cells in Layer 5 connect with subcortical structures. Cells in Layer 6 send their axons to the thalamus, indirectly providing input to the cortical area in which they lie, and to cortical regions linked functionally with their own.

1.4.2 Cerebral Cortical Vasculature

Neuronal survival is tied to the cortical vasculature, since it supplies oxygen and nutrients, and removes metabolic waste. The vasculature of the human cortex is similar in structure to that of non-human primates and rodents (Hirsch et al., 2012). The surface pial network has a 2D architecture (Nishimura et al., 2007) comprised of interconnected loops that protect against the effects of occlusion of single surface vessels (Blinder et al., 2010). The penetrating arterioles and venules dive into the cortex in a perpendicular direction, and connect the surface and subsurface networks. Branching from the penetrating arteries is a capillary network. This microvascular bed consists of a 3D tortuous mesh of small vessels where metabolite, gas, and heat exchange occur. Penetrating venules drain blood from the capillary bed back to the cortical surface. Below a more detailed description of the cortical vasculature is provided, based on the studies by Duvernoy et al. (1981) on the human cortex.
Generally the arterial network covers the venous network on the cortical surface. Pial veins are characterized by large diameters and numerous branches; arteries are smaller in diameter, and form a network with large meshes and right-angled branches. Arteriole diameters tend to narrow near their point of origin due to muscular sphincters that regulate arterial flow; venous diameter is constant. Numerous arterial and venous anastomoses exist, however arterio-venous anastomoses are not present. The absence of these anastomoses causes blood to first flow through the intracortical vascular network before being drained by the superficial veins. The density of superficial vessels varies among the cortical regions: it is highly vascularized in the occipital lobe (Duvernoy et al., 1981), but less vascularized at the top of the hemispheres near the median longitudinal fissure. No capillary network exists on the cortical surface.

The penetrating vessels, which direct blood to and from the capillaries, are distinguished by their depth of penetration. They are classified into 6 groups for humans (Duvernoy et al., 1981). Group 1 vessels feed/drain cortical layers 1 and 2, whereas group 2 vessels reach the 3rd layer. Group 3 vessels are the most numerous, and feed/drain layers 4 and 5, and the lower layer 3. Group 4 vessels reach the 6th layer and the inner limit of the subcortical white matter. Group 5 arteries and veins vascularize both the cortex and white matter, whereas group 6 vessels are large diameter arteries running through the grey matter without branching and exclusively vascularize the white matter.

Primary/superficial branches originate immediately following cortical entry by the penetrating arterioles and spread into cortical layers 1 and 2. These branches are infrequent. More frequently encountered are secondary or intermediate branches. These branches originate in layer 3 or at the layer 3/4 boundary, and usually turn towards the surface, ending in layers 1 and 2. They often coil around an artery, forming a rope-like structure. Tertiary, or deep branches arise at acute angles to the parent trunk and branch out in layers 4 to 6.
Duvernoy (1981) identified a laminar profile for the microvasculature, and found changes in vascular organization that paralleled the neuronal architecture. Four cortical vascular layers were identified. The first layer, located within the molecular layer, has the lowest vessel density and its meshes are largely oriented parallel to the surface. The 2\textsuperscript{nd} vascular layer is located in the superficial part of the pyramidal layer (layer 3), and is composed of parallel vessels perpendicular to the surface. The greatest vascular density is found in the 3\textsuperscript{rd} vascular layer, which traverses the bottom of cortical layer 3 and layers 4 (granular layer) and 5 (ganglionic layer). Its meshes are oriented in all directions. The 4\textsuperscript{th} vascular layer corresponds to the deep cortical cellular layers (layers 5 and 6), and vessel density dwindles until reaching the white matter.

### 1.4.3 Cortical Parcellation Schemes

Because cortical regions differ in many of their properties, a number of alternative methods exist for defining cortical region boundaries. These methods for defining boundaries are known as cortical parcellation schemes. In addition to highlighting variations across the brain, it is helpful to understand the different schemes since one must be selected to delineate cortical regions in the images. Cortical maps until the 1960’s were based on cyto- and myeloarchitecture, and the subdivisions produced with both techniques were comparable (Zilles and Amunts, 2010). At the synapse level, differences exist relative to the type and distribution of neuroreceptor binding sites (receptoarchitecture). In vivo autoradiography has demonstrated that different receptor types vary in relation to their concentrations between different cortical regions, and their distribution across cortical layers in a given region (Cloutman and Ralph 2012). Similar to cyto- and myeloarchitecture, neurotransmitter specificity is implicated in the functioning of neuronal populations, and studies have shown a strong correlation between cytoarchitectonic and receptoarchitectonic boundaries (Cloutman and Ralph 2012).
Cortical maps may possess a functional basis. They incorporate results of studies based on electrophysiology, induced gene expression, optical imaging of intrinsic signals (detect changes in optical properties of active tissue), and calcium imaging which link the anatomy to external stimuli. Structural white matter connections are a type of long range “connectivity” that may be detected in vivo, and patterns of functional connectivity within the cortex have identified regions of functional specialization (Cohen et al., 2008). Grey matter connection (connections between neurons within the cortex) provides an alternative viewpoint to cytoarchitecture. Cytoarchitecture determines a region’s local processing abilities, whereas connectivity impacts flow of information from one area to another. Brain regions performing unique functions require different neural connectivity architectures. Imaging these connections, with diffusion magnetic resonance imaging for example, render this a possible parcellation technique. This type of function-based parcellation scheme may shed light on higher cognitive functioning which depends on interactions between different brain regions (Cloutman and Ralph, 2012).

The Allen Reference Atlas (Lein et al., 2007), which was used in this study, uses Nissl-staining (cytoarchitecture) to delineate the cortical regions. Reasons for its selection are highlighted in Chapter 2.

1.4.4 Mouse as a Model of Human Neocortex

Superficially the mouse cortex is similar to the human in protein sequences, growth patterns, and a common organization structure. The cellular layers are homologous in terms of their cellular constituents and projection targets. However, differences exist, and must be considered when extrapolating data from mice to humans.

Functionally, the human cortex is more involved in purposeful behavior; mice use the cortex for guidance but it exerts less direct control on movement. The creases and grooves in the human cortex, and larger proportion of white matter, suggest a complexity of neuronal connections absent in the mouse.
The primate neocortex scales differently with brain and body weight, suggesting differences in brain growth regulation and development. Bipolar interneurons are more common in mice, and double-bouquet neurons are found in humans, not mice. However, similarities in neuronal density in multiple areas in rodents, cats, and primates, indicate most species differences may be attributable to neuronal connections as opposed to cellular composition. The mouse brain possesses thinner axons and dendrites, with possibly fewer synapses as well. In humans, the cortical layers 2 and 3 are separate, but in mice are combined as one layer. (Watson et al., 2012). These differences may affect the vasculature on a fine scale, such as capillary connectivity and density. However, studying the mouse may still provide some insight into how metabolic demands and cellular properties impact the vascular architecture, as many of the neurons, cortical regions, and metabolic requirements are similar between the species.

### 1.5 Physics and Technology

This section focuses on optical microscopy imaging. Initially, the interaction of light with tissue is considered, since these interactions affect imaging depth in tissue. 2-photon microscopy instrumentation is discussed, together with a description of methods for increasing the imaging depth in tissue. Optical clearing facilitates increased imaging depth, though due to refractive index mismatches between BABB (1.56) and water (1.33), optical aberrations, in the form of spherical aberrations, are present in the images. Spherical aberrations and their effect on image quality are discussed. Methods for minimizing the impact of spherical aberrations, particularly deconvolution as used in this work, are advanced. Finally, a brief description of the segmentation algorithm used for data analysis follows.

#### 1.5.1 Light Interaction with Tissue

Optical imaging techniques are usually limited to the pial layer because the intensity of light is strongly attenuated with depth. In most tissues, the absorption is negligible in comparison with the degree of
scattering, especially in the near-infrared wavelength range (Helmchen and Denk, 2005). Scattering occurs when the light ray changes direction, and is elastic if no energy is lost. Elastic scattering is the result of refractive index heterogeneities (Helmchen and Denk, 2005) which are strong in tissue owing to the many components of cells with different refractive indices such as fibrils, cytoplasm, nucleus and cell membranes, and the various organelles (Tuchin, 2006). The probability and angular distribution of scattering depends on refractive index variation, object size, and wavelength. In Rayleigh scattering, which is proportional to the $4^{th}$ power of the light wavelength, the wavelength is much larger than the size of the object. Examples include scattering off of collagen fibrils and macromolecular aggregates. Scattering in this case is isotropic, or directed equally in all directions. Mie scattering occurs when the diameter of the scattering particle is on the order of the wavelength, as with mitochondria and vesicles, and is mainly directed in the forward direction. Mie scattering decreases with wavelength, though not to the same degree as with Rayleigh scattering, and is proportional to the square of the ratio of refractive indices of the surrounding media and scatterer (Pawley, 2006). Measurements in in vivo brain grey matter indicate a mean free path of 200 µm at 800 nm (Helmchen and Denk, 2005). The mean free path is a measure of scattering strength and is the average distance between 2 scattering events. The imaging depth may be increased via minimizing refractive index mismatches and selecting longer excitation wavelengths.

1.5.2 2-Photon Microscopy

2-photon fluorescence microscopy was developed by Denk et al. in 1990 and was first used to image chromosomes in dividing cells. It is a 3D imaging technique with typically about 1 µm resolution. The in vivo imaging depth is about 700 µm, and the technique has found a wide range of applications in neuroscience, including in vivo vascular imaging, and functional imaging of neurons and their network
activity (Helmchen and Denk, 2005). Below is a discussion on the advantages of 2-photon imaging and the instrumentation that makes it viable.

**Fluorescence**

Fluorescence is the means by which a 2-photon microscope set-up detects signals from biological tissue. Conventional fluorescence is stimulated by absorption of a single photon by a fluorophore, which raises an electron in the fluorophore to a higher energy electronic, and/or vibrational state. In the unstable excited state, the molecule relaxes to the ground vibrational state. When the electron returns to the ground electronic state, it emits a photon of slightly longer wavelength than was absorbed. As a consequence of the Heisenberg Uncertainty Principle, where for 2-photons of a certain energy arriving at the same location there is a limit in the accuracy with which their arrival times may be measured, one atom or molecule can absorb 2 photons in the same quantum event within $10^{-16}$ – $10^{-17}$ s (Diaspro et al., 2005). This process is termed 2-photon fluorescence. Each photon has approximately half the energy required to induce an electronic transition, but since they are absorbed together, the result is the same as if the photons combined their energy and became a single photon. Quantum mechanically, a single photon excites the molecule to a virtual intermediate state, and is brought to the final excited state by absorption of the 2nd photon (So et al., 2000).

2-photon absorption has several advantages in comparison with single photon absorption. Near infrared light (700-1000 nm) penetrates deeper into scattering tissue (Helmchen and Denk, 2005). Penetration is deeper because longer wavelengths are less scattering, and most tissue has reduced absorption in the near infrared. Since two photons are absorbed, the excitation intensity depends quadratically on the incident light intensity. This implies that 2-photon absorption is strongest in the focal region, and minimizes the signal from out of focus planes. In confocal microscopy, where 1-photon absorption occurs in the focal region, a pinhole is used to exclude light from out of focus regions. This method limits
the signal from deeper in the tissue since most photons emitted by the fluorophore are scattered, and rejected by the pinhole. 2-photon excitation obviates the need for a pinhole, allowing for increased signal from deep in tissue. The localization of excitation is maintained in highly scattering tissue due to a insufficient density of scattered photons to induce 2-photon fluorescence, provided sufficient density of photons at the focus is maintained. An additional advantage to the localization is the reduced photointeraction region which lessens the total specimen photobleaching and phototoxicity (So et al., 2000; Denk and Svoboda, 1997). For example, in a comparison of 2-photon and confocal calcium imaging of living cells in culture, Sako et al. (1996) found the photobleaching rate of the calcium indicator to be decreased by a factor of 2-4 for 2-photon imaging.

Instrumentation

(i) Laser sources

2-photon absorption is a 2nd order process with a probability cross-section of order $10^{-50} \text{cm}^4\text{s}$. A high photon flux, using ultrashort pulsed lasers, is required to generate sufficient absorption for detection of fluorescent emissions.

The most widely used laser source is the femtosecond titanium-sapphire (Ti-Sapphire) system. It emits light between 700 and 1000 nm, enabling excitation of most fluorophores. Its 80 MHz repetition rate is comparable to common fluorescent lifetimes, balancing excitation efficiency and saturation onset (Helmchen and Denk, 2005). Short pulse widths on the order of 100 fs, achieved with mode-locked lasers, prevents overheating or vaporization of tissue since power is delivered over a short time. The average power of the laser is 100 mW, while the peak power is 15 kW (Lindvere, 2011).
(ii) Scanning fluorescence microscopy optics

The laser beam is expanded using a telescope preceded by a laser intensity modulator. The beam is scanned by an xy-deflection module such as a pair of galvanometric scanners. It is further expanded by the scan and tube lens to fill the back aperture of the microscope objective, which focuses light onto the sample (Helmchen and Denk, 2005). The xy-galvanometers provide lateral focal point positioning, and raster scanning in 3D is performed via an objective positioner that translates the focal point axially (So et al., 2000).

(iii) Fluorescence detection system

The fluorescence emission is collected by the same imaging objective used to focus the light, reflects off the dichroic mirror along the emission path, and is directed to the detector system. Typical photodetectors are photomultiplier tubes (PMTs), avalanche photodiodes, and charge-coupled-device cameras (CCDs), of which PMTs are most commonly used. PMTs are low cost and sturdy, have large active areas for collecting scattered light from a wider field of view, and are relatively sensitive (So et al., 2000).

1.5.3 Optimization for Deep Tissue Imaging

This section discusses methods for maximizing the imaging depth for 2-photon microscopy. This allows for visualization of the deeper layers (4-6) of the neocortex.

Excitation wavelength

To generate signal, light has to reach the focal point with minimal scattering. This is achieved by using less scattering, longer wavelengths. The 2-photon cross-section as a function of wavelength must be considered and less laser power is available at very long wavelengths (Helmchen and Denk, 2005).
Pulse width

Shorter pulse widths with high instantaneous power maximize fluorescence generation. Pulse broadening must be considered when shorter pulse widths are used. Pulse broadening is when longer-wavelength (red) components of the pulse spectrum travel faster through material than shorter-wavelength (blue) components, resulting in lengthening, or ‘chirping’, of the pulse. This phenomenon, termed group delay dispersion, may be compensated by providing the short-wavelength components with a head start (a negative ‘prechirp’) so that red and blue components arrive at the focus simultaneously (Helmchen and Denk, 2005). Prechirping is accomplished with grating or prism elements that separate the different wavelength components. These wavelength components then travel different path lengths and are recombined (Helmchen and Denk, 2005). An issue with prechirping is that additional optical elements in the light path may lead to power losses.

Minimization of tissue scattering via optical clearing

Even with optimal excitation wavelengths, pulse widths, and use of a regenerative amplifier, the imaging depth achievable is up to 1 mm in the neocortex (Theer et al., 2003). Regenerative amplifiers amplify single pulses from a mode-locked oscillator via optical switches which insert a pulse into a cavity and remove the pulse from the cavity precisely once it has been amplified to a higher intensity. For deep tissue images obtained with the use of a regenerative amplifier, contrast at 800 µm is weak and beyond 1 mm only vessels and cells with diameters greater than 10 µm are visualized (Theer et al., 2003). Because tissue is composed of materials with a variety of refractive indices, light emitted from deeper in the tissue is significantly scattered and much of it does not reach the detector. This process results in weak signals emitted from deeper in tissue, and limits the imaging depth achievable. If the tissue were transparent, then the scattering of emitted light is minimized, which should result in increased imaging depth. Optical clearing of tissues via organic solvents is emerging as a method for imaging thick tissues.
(Erturk et al., 2012) by inducing transparency in the tissue. Tissues are made transparent by matching the refractive indices of different tissue layers to the solvent. This minimizes light scattering in tissue, and the light travels relatively unobstructed through the different tissue layers.

The first step for clearing specimens in organic solvents is dehydration, where the water, which is insoluble in organic solvents, is removed from the tissue and replaced with alcohol, usually methanol, which is soluble in organic materials. The dehydrated material is impregnated with an optical clearing agent such as methyl salicylate, BABB, or dibenzyl ether (DBE). BABB, with refractive index 1.56, and DBE, with the same refractive index (Becker et al., 2012), produce specimens with the highest degree of transparency. Parra et al. (2010) cleared mouse organs with BABB and obtained penetration depths in excess of 2 mm with a 2-photon microscope.

This clearing procedure does not allow for deep visualization of tissue containing a high degree of lipids and a sturdy extracellular matrix (Becket et al., 2012 (2)), such as the subcortical white matter. Methods have been devised for visualization through the cortical depth and deep into the white matter. Becker et al. (2012 (2)) replaced alcohol dehydration with dehydration in tetrahydrofuran (THF), which more readily dissolves lipids. Clearing THF-dehydrated specimens in BABB resulted in fully transparent spinal cords (Becker et al., 2012 (2)). Recently, Chung et al. (2013) developed a clearing method, termed CLARITY, where hydrogel monomers are infused into the mouse brain, and application of an electric field across the sample results in lipids being transported out of the tissue. Incubation of the brain in FocusClear (CelExplorer Labs) or 85% glycerol produces fully transparent tissue, in both the grey and white matter.
1.5.4 Aberrations in Microscopy

Under ideal imaging conditions, such as no refractive index mismatches, the image of a point source object (the Point Spread Function, or PSF) is the diffraction pattern produced when the lens converts a planar wave into a convergent spherical one. Under these conditions, the image is diffraction limited and aberration free (Gibson and Lanni, 1992). When the light passes through layers whose refractive indices vary, aberrations are introduced which distort or change the shape of the wavefronts and shift their focal position. Since the light rays emanating from the objective lens do not intersect the interface of the layers at the same angle, the peripheral and paraxial rays are not focused at the same point along the optical axis. This type of aberration is termed ‘spherical aberration’. These aberrations cause a focal shift (axial scaling of the image) and a spreading of the focus both in the lateral plane and along the optical axis, which results in reduced resolution and signal intensity. These effects are proportional to the focusing depth (Booth, 2007). The PSF is distorted geometrically as well, and displays asymmetry and characteristic flares emanating from its edges. Multiphoton processes are strongly influenced by aberrations, since the absorption probability depends non-linearly on the focal intensity. The signal intensity may be restored by increasing the illumination laser power, but this does not restore the resolution.

Image quality may be improved by manipulating the degree of spherical aberration. Water-immersion microscope objectives are often equipped with adjustable correction collars. These collars move the objective lens elements so that peripheral and paraxial rays of light form a tight focus after traveling through materials with different refractive indices than water. Typically correction collars adjust for varying coverslip thickness. Muriello and Dunn (2008) were able to restore vertical symmetry of images taken of 0.5 um fluorescent beads after focusing through a coverslip of 0.17 mm thickness with a correction collar. Helmuth et al. (1988) showed that overcorrecting for the coverglass thickness allows
for compensation of spherical aberration at certain depths in a specimen with refractive index
mismatch.

These methods use static means to correct for spherical aberrations at specific depths, not dynamic compensation while the beam is focused deeper into the sample. They typically have minimal impact on signal intensity or PSF more than 30 µm deep into a specimen (Lo et al., 2005). Wavefront distortions may be corrected during imaging with adaptive optics. A waveshaping element, such as a deformable mirror, can correct for specimen-induced aberrated wavefronts detected with a wavefront sensor (Neil et al., 2000).

_Deconvolution in microscopy_

Adaptive aberration corrections lose effectiveness through the depth of the sample. PSF distortions become more prominent and the deformable mirrors are difficult to engineer to deform into the required shape to correct the PSF (Sherman et al., 2001). A method which can adequately process images through the sample depth is desirable. This is achievable through image deconvolution.

Every imaging system has a unique PSF determined by the optical characteristics of the sample, objective lens, wavelength, and microscope. 2-photon microscopes have a PSF shaped as an ellipsoid along the optical axis. Any object can be considered a collection of point sources, or a summed collection of PSFs, where each PSF is centred according to the location of the point source.

Mathematically, this operation is called convolution (Biggs, 2004). The image presented to the user is not the ideal image free from aberrations, but a blurred image which is the ideal image convolved with the spherically aberrated PSFs. If the PSF is known and does not possess any zeros, it is theoretically possible to “work backwards” and restore the image quality, or obtain the ideal, unaberrated image. This operation is deconvolution.
Constrained iterative algorithms for deconvolution are popular choices for 3D optical data sets, and were implemented in this thesis (see Chapter 2). Two subsets of constrained iterative algorithms are non-blind and blind deconvolution. In non-blind deconvolution, the PSF is measured by the user and submitted to the computer. An initial estimate of the object is made, which is usually the raw image. This estimate is convolved with the PSF, and the resulting “blurred estimate” is compared with the raw image. This comparison is used to compute an error criterion that estimates the similarity of the blurred to raw image. This error criterion adjusts the estimate so that the error is reduced. A new iteration takes place where the new estimate is convolved with the PSF, and the process is repeated until the error criterion is minimized (Wallace et al., 2001). One common constraint is smoothing, or regularization. As iterations proceed, noise is amplified and regularization suppresses this noise with a filter (Wallace et al., 2001). An additional constraint is non-negativity, where any pixel value that becomes negative during the iteration process is automatically set to 0. This is done since an object with negative fluorescence is unrealistic. In imaging beads to characterize the PSF (a requirement for non-blind deconvolution), a high signal to noise ratio is required, and sometimes multiple beads must be averaged. The bead-imaging process may be time consuming, and a single measured PSF to characterize an entire optical system is often not accurate in the case of spherical aberrations, or where the PSF may vary across the field of view. However, an advantage to measuring the PSF is that a theoretical PSF, though mostly correct, will be slightly different than that of the actual optical system as dirt or scratches on the lens distort the PSF and are difficult to take into account via theoretical modelling.

Blind deconvolution does not require the user to submit a PSF. It is iterative, and both the object and PSF are estimated. In this approach, the object is estimated and convolved with a theoretical PSF calculated given the properties of the optical system. Parameters included in the theoretical model are the immersion medium of the objective lens, coverglass thickness, wavelength of light used for excitation, numerical aperture of the objective lens, and the refractive index of the object medium. The
resulting blurred estimate is compared to the raw image. The correction is computed, and this correction is applied to produce a new estimate. The same correction is applied to the PSF, resulting in the PSF and object estimate being updated together. Blind deconvolution does not require acquisition of a high quality PSF. It corrects for spherical aberration since it is adapted to the data. If the image is subdivided during processing, a different PSF may be determined for each region taking into account axial elongation with depth (Biggs, 2004). The degree of axial elongation depends on the degree of refractive index mismatch between the lens and specimen immersion mediums. The axial resolution of heavily aberrated PSF’s is roughly proportional to focusing depth (Booth and Wilson, 2001). In imaging through a specimen immersed in BABB with a water immersion microscope objective (numerical aperture of 1.05, working distance 2 mm), the axial FWHM at the specimen surface is 1.4 µm (Lindvere 2011), and increases linearly to about 20 µm at a focal depth of 2 mm into the specimen (experiments conducted by author of thesis).

1.5.5 Vessel Segmentation

Blood vessels may be approximated as trees of branching tubes (Pries and Secomb, 2004). To perform a quantitative analysis on large vascular networks similar to Cassot et al. (2006), where properties of these networks such as vessel density, length density, and capillary orientation were assessed, the 3D geometry and the tubes themselves must be extracted. This extraction process is referred to as segmentation. The segmentation algorithm used in this thesis is based on that of Fridman et al. (2004), and is described in Rennie et al. (2011). It finds the centerlines of branching tubular objects in 3D data sets, and calculates the radius of each tube. Algorithms based on finding centerlines are less sensitive to noise since the signal is strongest in the vessel centre. A digital marker is placed in a vessel to define a segmentation starting point. The centerline is represented by discrete medial atoms. 8 spokes extend from the vessel centre to the implied vessel boundary. The intensity gradient in each spoke direction,
from the centerline to the spoke tip, is calculated, and summed for an atom. When the orientation, position, and radius of the atom correspond to the vessel direction, centre point, and radius of the lumen respectively, the sum is minimized and these parameters (direction, centre point, and lumen radius) are recorded. The parameters of the next atom in the vessel are initially estimated from the parameters of the preceding atom, and then optimized for position and radius. Branch points are recorded when one of the spoke directions has a much smaller image gradient than the others (Rennie et al., 2011). Regularization prevents the algorithm from calculating an atom position or radius too far off the initial estimate.

The algorithm generates the centerlines of the vessel network. Each vessel segment is sampled along its midline by vertices. Contained at each vertex is the vertex position, tangent of the vessel, radius of the vessel, and the other vertices to which that vertex is connected. Examples of quantitative parameters that may now be extracted include vessel length, density, branching angles, tortuosity, and vascular volume. This algorithm provides the base for comparison of cortical regions.

1.6 Summary of Introductory Chapter

This chapter provided the motivation for comparing vasculatures across the cortex, and outlined general characteristics of the cerebral vasculature and cortex. It described the role that a new imaging methodology may serve in making this comparison.

Chapter 2 will focus on the main aspects of this methodology, such as specimen preparation and imaging, and development of vessel network analysis tools. Chapter 3 will analyze the benefits of the technique, while considering its limitations and possible future applications.
Chapter 2

This chapter presents the novel imaging methodology briefly described in Chapter 1. It summarizes the rationale and benefits underlying development of a new imaging technique (Section 2.1). Described in detail are the specimen preparation protocols and the data acquisition procedure for acquiring highly detailed images of the cortical vasculature (Section 2.2). The analysis tools that were established and built-upon as part of this research are detailed (Section 2.3). The main results of Sections 2.3 and 2.4 are then summarized (Section 2.4) to highlight the major outcomes of the research. This summary serves as a lead-in to Chapter 3, where the advantages and limitations of the imaging methodology are discussed. In each section, reasoning behind each step involved in the imaging and analysis processes are provided.

2.1 Justification for a New Imaging Methodology for Visualization of the Mouse Cortical Vasculature

The cerebral cortex is a thin layer of tissue covering the cerebral hemispheres. It is responsible for diverse functions such as movement, speech, vision, thought, hearing, and emotion. The cortical vasculature is a highly ordered network designed to transport materials such as oxygen and glucose throughout the cortex. The large diameter pial vessels are the most efficient means of blood transport over long distances as their larger diameters minimize resistance to fluid flow (Secomb and Pries, 2011). These vessels form interconnected loops (Blinder et al., 2010) to protect against the effects of single-vessel occlusion. Emerging from the pial vessels and entering the cortex at right angles are the penetrating vessels, which connect the pial network with the subsurface microvasculature. In the microvasculature, oxygen, glucose, and other metabolites are delivered to neurons to perform their metabolic functions.
The efficient delivery of oxygen and glucose to neurons is dependent upon the 3D arrangement of blood vessels in space. This 3D architecture affects blood flow distribution, and nutrient and oxygen delivery to neurons. Each cortical region possesses different functions, and areas differ in metabolic activity, nerve fibre distribution, and cell types (Sokoloff et al., 1977; Wree et al., 1990; Lewis et al., 1977). Fine differences in the vascular networks may exist across the brain that reflect an area’s unique metabolic demands and cellular architecture. For example, Borowski and Collins (1989) found a positive correlation between capillary density and glucose consumption, suggesting a relationship between vessel density and metabolic activity. Similarly, Zheng et al. (1990) and Keller et al. (2011) found the total number and length density of microvessels to be greater in highly active functional modules in primate visual cortex. Studying vascular patterning in the cortex may provide insight into the arrangement of networks to meet a tissue’s metabolic demands, while shedding light on the structural organization of the cortex (Zheng et al., 1990).

Most studies comparing the vasculature between brain regions have relied on corrosion casting and 2D histology techniques. In corrosion casting, the vessel lumen is filled with a casting polymer, the tissue corroded, and the resulting cast visualized with a Scanning Electron Microscope (SEM) (Heinzer et al., 2006). This technique is effective for a qualitative assessment of microvascular organization, and for distinguishing between arteries and veins (Weber et al., 2008). Vessels may fall off the cast lost during the corrosion process as the tissue no longer supports the vessel. In addition, unless the cast is dissected, structures deep within the brain are difficult to visualize. Accurate quantitative metrics, such as vessel length, are challenging to obtain, and depend upon the angle at which the cast is viewed (Minnich et al., 2000). This can be an issue when comparing vascular lengths between regions, as comparisons between regions may not be meaningful since the cast will not be viewed at precisely the same angle for each region.
In using histology, the vasculature is stained and the brain subsequently sectioned for visualization with light microscopy. These images possess a high resolution, yet the spatial extent and depth penetration are limited (Moy et al., 2013; Hirsch et al., 2012). Furthermore, tissue slicing induces distortions in the tissue and vasculature (Weber et al., 2008).

Corrosion casting and histology fail to capture the 3D nature of the vascular architecture. 3D digital data sets enable the connectivity and shapes of vessels to be analyzed, as entire vessel segments typically extend beyond a thin tissue section. These properties are important since they impact the overall resistance of a network to blood flow as longer vessels with smaller diameters confer more resistance to flow. As bifurcations at the end of a vessel segment provide alternate/additional routes for blood to flow through a network, and each of the segments at a bifurcation possess their own individual resistance to fluid flow, the connectivity of a network (in addition to the shapes of blood vessels) impact fluid flow, and hence nutrient and oxygen delivery to tissue. A comparison of the 3D architectures between regions may provide insight into interpretation of the Blood Oxygenation Level Dependent signal (BOLD) in functional Magnetic Resonance Imaging (fMRI). This signal is related to the blood volume, blood flow, and blood vessel geometry (Menon and Kim, 1999). It is difficult to directly compare fMRI results between different areas due to differences in vascular density and architecture (Lorthois et al., 2011). Information on the vascular architecture in different regions may render comparison possible.

The first step to quantifying the vasculature in different cortical regions is to develop an imaging methodology for imaging large portions of the cortical vasculature. It is important to image large tissue volumes as the vasculature varies significantly within a brain region (Keller et al., 2011), and large field of view images are necessary to capture this variation. Ideally the technique will provide resolution for visualization of blood vessels of all sizes, including capillaries. As capillary density has been found to be proportional to tissue metabolic demands (Keller et al., 2011), information on these vessels may provide
insight on the relationship between tissue function and vascular architecture. The technique must allow imaging deep into the cortex to obtain vascular information on the cortical layers with highest metabolic activity (layers 3-5), as this is where many of the vessels are found. However, these imaging depths must be achieved without dissecting the tissue, as dissection deforms the vasculature at the cutting boundaries. The images acquired should be 3D so that the angioarchitecture is accurately depicted, and so that image segmentation techniques may be incorporated for rigorous quantitative analysis.

While 3D imaging methodologies for capillary visualization in the cortex have been developed and described, they each require the brain to be sectioned, or small blocks of tissue to be removed to enable acquisition of high resolution images deep below the cortical surface. For example, Moy et al. (2013) optically cleared 1 mm-thick coronal slabs of mouse brain tissue in FocusClear (CelExplorer Labs, Hsinchu, Taiwan), a dimethyl sulfoxide based optical clearing agent, and imaged the vasculature in these slabs with a confocal microscope. It was necessary to section the brain tissue to visualize the vasculature deep in the cortex as the imaging depth achievable with FocusClear is only 850 µm, while some cortical regions possess a thickness up to 1.5 mm (Lerch et al., 2008). Similarly, confocal microscopy on opaque human brain tissue constrained Lauwers et al. (2008) to imaging 300 µm thick slabs due to limited depth penetration. Though micro-CT enables capillary visualization, the sample volume is small (approximately 1 mm³ in size), and is dissected from the tissue prior to imaging. In addition to inducing deformation artifacts at the cutting boundaries, it is difficult to localize tissue that has been dissected (Heinzer et al., 2006). Though synchrotron radiation micro-CT has produced images of bone at 1.4 µm voxel size with a field of view of 2.8 x 2.8 x 2.2 mm (Larrue et al., 2011), this field of view is still not sufficient to encompass entire cortical regions.

This paper presents a novel methodology for imaging the vasculature of large cortical regions in 3D of an intact mouse brain. The methodology involves the following components: The brain is perfused with a
fluorescent contrast agent dissolved in a low viscosity liquid plastic that solidifies inside the vasculature, stabilizing the contrast agent. The brain is fixed, then rendered transparent via soaking in a chemical solution of by volume 1:2 benzyl alcohol:benzyl benzoate (BABB) that possesses a similar refractive index to the tissue membranes. Imaging through the whole cortical depth is performed with a 2-photon microscope. Separate single field-of-view images are then stitched together to produce an image of a large volume of vasculature at the same resolution. The Allen Reference Atlas (ARA) (Lein et al., 2007) is next registered to the 2-photon data for delineation of the cortical regions. Quantitative metrics are extracted from the different regions using an automatic vessel segmentation algorithm. These metrics are compared with those obtained by other investigators to validate this technique, and are found to be in agreement. Since this methodology has the ability to visualize all vessel types in the cortex, and provides reasonable estimates of quantitative parameters, it shows strong potential for quantitative analysis of normal and abnormal vascular architecture.

2.2 Specimen Preparation Protocols and Imaging

2.2.1 Perfusion of the Vasculature with a Fluorescent Contrast Agent

For ex vivo vascular visualization with a 2-photon microscope, the vessels must be perfused with a fluorescent contrast agent. The contrast agent Nile Red was selected due to its intense fluorescent emissions. Its quantum yield, which is the probability of fluorescent emission upon absorption of a light photon, is listed as 0.7 (www.anaspec.com). To render the vasculature permanently fluorescent, Nile Red was dissolved in a liquid resin, Mercox (Ladd Research, Williston, VT, USA), and hardener (Ladd Research) that causes the Mercox to solidify inside the blood vessels. This prevents the Nile Red from diffusing out of the vasculature. Mercox was selected as it has a low viscosity and has been previously shown to fill brain capillaries (Dickie et al., 2006). A description of the perfusion protocol is provided below.
6 male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA), weighing 20-25 g, aged 10-12 weeks, were anaesthetized via an intraperitoneal (IP) injection of 100 µg ketamine per gram of body weight (Pfizer, Kirkland, QC, Canada) and 20 µg of xylazine per gram of body weight (Bayer Inc., Toronto, ON, Canada). The chest of each mouse was opened, and a 24-gauge IV catheter (Becton Dickinson Infusion Therapy System Inc., UT, USA) was inserted into the left ventricle, and a slit was made in the right atrium for outflow of blood.

The mice were perfused with 30 mL of heparinized (5U/mL) 0.1 M phosphate buffered saline (Wisent Inc., St-Bruno, QC, Canada) at a constant flow rate of 2.5 mL/min. During this process, a solution was prepared containing 15 mL of a 1:1 by volume mixture of BABB : Mercox (Ladd Research, Williston, VT, USA). The Mercox was diluted with BABB prior to perfusion to render the overall refractive index of the perfusion material as close to BABB as possible, as BABB is the chemical in which the specimen is later incubated. This ensures that during imaging with 2-photon microscopy, the refractive index variation within cortex is minimized. This minimizes tissue light scattering and potentially imaging artifacts at blood vessel-tissue boundaries. 10 mg of fluorescent technical grade Nile Red (N3013, Sigma-Aldrich, St. Louis, MO, USA), grounded into powder form, was dissolved in the BABB-Mercox solution, and filtered through 0.8 µm pore size filter paper (PN 4618, Pall Corporation, Port Washington, NY, USA) to prevent clumping of the powder, which may block complete capillary perfusion. A quantity of 0.5 g of benzoyl peroxide catalyst (the hardener) was then added to the mixture, stirred for approximately 1 minute, and perfused at a constant flow rate of 2.5 mL/min through the mouse. 0.5 g of hardener (benzoyl peroxide catalyst) was added since this quantity causes polymerization of the Mercox in about 5-6 minutes, which is ample working time for perfusion. Further, the polymerized mixture is sufficiently hard to prevent diffusion of the contrast agent from the vasculature. Once the 15 mL of perfusant had passed through the mouse, the mouse was placed in a Styrofoam container filled with ice for 20 minutes. Storing
perfused animals on ice has been performed in the past (Dickie et al., 2006) to prevent damage to tissue during the exothermic curing process.

### 2.2.2 Fixation, Dehydration, and Optical Clearing

To increase the transparency of cortical tissue, it was optically cleared in BABB. Clearing with BABB and imaging with a 2-photon microscope has previously been shown to result in imaging depths in excess of 2 mm (Parra et al., 2010). Since the maximum cortical depth in the mouse brain is approximately 1.5 mm (Lerch et al., 2008), clearing in BABB provides the ability to image through the whole cortical depth.

Upon removal from the Styrofoam container after perfusion, external soft tissue was dissected from the mouse skulls. These skulls were fixed for 24 hours at 4°C in 1% PFA (Electron Microscopy Sciences, Hatfield, PA, USA). They were transferred for 1 day to 0.1 M PBS, then the skull surrounding the brain was dissected.

The brains were dehydrated in a methanol series consisting of the following 0.5 day steps: 25 %, 50 %, and 75 % v/v in distilled water. Dehydration is necessary as BABB is immiscible with water, but will penetrate methanol-filled tissue. They were transferred for 2 days to 100 % methanol, and stored in BABB until imaging with the 2-photon microscope. Transparency was typically achieved about 3-6 hours after BABB incubation. The dehydration procedure employed has been shown to cause a mean 16 % isotropic, by volume tissue shrinkage (Bucher et al., 2000). This shrinkage is not affected by filling the vasculature with Mercox.

### 2.2.3 2-Photon Microscopy and Data Acquisition

Of the 6 specimens perfused, 3 were imaged with the 2-photon microscope, and 3 were used to qualitatively assess perfusion quality. Filling of the large surface venous vessels, such as the superior sagittal sinus which runs along the superior midline, is one possible indicator of a good perfusion as the
perfusant must first pass through the capillary network before filling the venous vasculature. Perfusion quality was further assessed by determining whether the imaged network possessed features typical of capillary networks, such as numerous interconnected loops. A more quantitative assessment of perfusion quality was calculated by segmenting the vasculature (see Vessel Tracking, 2.3.1) and comparing measures such as the total length of capillaries per unit volume with those reported by other investigators (see Extracting Quantitative Parameters, 2.3.5). Each of the 3 brains imaged was super-glued to the bottom of a glass well (Loctite, Westlake, OH, USA). The well was filled to the brim with BABB, and a No. 1 glass coverslip was placed over the well. A drop of water was inserted on the coverslip surface. The well was placed on a platform that was controlled in the xy-plane (perpendicular to the optical axis of the microscope objective) with a set of Vernier calipers.

2-photon fluorescence microscopy was performed on a twin FV1000 Multiphoton Excitation Microscope (Olympus Corp., Tokyo, Japan) using a water-immersion objective (25x, 1.05NA, 2 mm working distance). The objective was lowered into the water drop placed on the coverslip for imaging. The excitation wavelength used to excite the Nile Red was 800 nm. The emission filter bandwidth spanned 570 to 620 nm. Images with voxel size 0.994 µm x 0.994 µm in the xy-plane and 3 µm along the optical axis, were obtained up to 1.8 mm below the coverslip. The spacing in the xy-plane is determined by the angle of rotation of the scanning mirrors after a signal from a pixel in the plane is recorded. This decides the deflection angle of the laser beam, and hence the horizontal distance moved by the beam the spot of the laser between sampling points. The spacing between each xy-plane (3 µm) is the vertical distance moved by the objective lens after a given plane is scanned. The full 2 mm working distance available was not imaged due to concerns of the objective making contact with the glass coverslip. The image field of view was 508 µm x 508 µm.
For each of the 3 brains, images of the perfused vasculature through the whole cortical depth were acquired with the brain intact. These were obtained by producing an image at the left side of the brain, and moving the sample laterally by about 0.4 mm so that there was about a 20% overlap between the two neighbouring images. A total of about 16-18 overlapping images were produced ranging in space from the left side of the brain to the right side (approximately 8 mm). This results in an image with a field of view of approximately 0.5 mm in the cranial-caudal direction, and approximately 8 mm laterally.

Before stitching, each of these sub-images was deconvolved using commercial deconvolution software (AutoQuant X3, MediaCybernetics, Rockville, MD). The adaptive blind deconvolution algorithm was used, which draws upon the techniques of Maximum Likelihood Estimation and Constrained Iteration (Holmes, 1992) to deconvolve the image without requiring submission of a point spread function. Blind deconvolution was chosen over non-blind deconvolution as it has the ability to take into account the axial elongation with depth caused by the spherical aberration. Deconvolution did not render the data isotropic. However, it decreased the signal of the flares emanating from the vessels, caused by the flares in the spherically aberrated PSF. The amount of spherical aberration could be further reduced by increasing the number of iterations required by the algorithm for convergence. However each iteration results in a decrease in the contrast-to-noise ratio of smaller, weak signaled vessels, which makes these vessels more challenging to segment. Thus, there is a trade-off between sharpening of the PSF and signal from smaller capillaries. A further trade-off in deconvolution algorithms exists between resolution restored and amplification of noise. The optical transfer function (OTF) is the Fourier Transform of the PSF. Deconvolution strongly suppresses any details in an image with spatial frequencies outside the OTF (Pawley, 2006). These details may arise due to the effects of Poisson noise on the signal intensity in individual voxels. These details are suppressed at the cost of restoring a higher resolution to the deconvolved image. Stitching of the deconvolved sub-images into one large data-set was accomplished.
using the freely-available XuvTools (Emmenlauer et al., 2009). One of these large volume data sets is shown in Figure 2.1 (A).

After imaging with the 2-photon microscope, the brains were imaged with Optical Projection Tomography (OPT) using a custom-built OPT scanner described in Gleave et al. (2012), and Wong et al. (2013). The OPT image was used to help delineate cortical regions in the 2-photon data (see Section 2.2.4 for more details). A λ=500 to 550 nm excitation filter and λ=575 to 625 nm emission filter were used during OPT imaging. The voxel size was about 12 µm after reconstruction, and the field of view encompassed the entire mouse brain. The OPT images were obtained as an intermediate step in a registration procedure described in the next section.
Figure 2.1. (A) Maximum Intensity Projection (MIP) through the 0.5 mm thick deconvolved 2-photon data set analyzed in this thesis. Scale bar = 1 mm. Colour map ranges from 0 (black) to 1 (bright yellow). (B) Seed placement based on local signal intensity maxima. Each tiny green dot in this image is a seed. A number of the larger surface vessels and penetrating vessels visible in (A) are also visible in (B). There are almost 200 000 seeds in the entire image. Scale bar= 1 mm.
2.2.4 Delineation of the Cortical Regions in the 2-Photon Data

To compare the angioarchitectures across cortical regions, a method was devised for determining the cortical regions to which the vessels in the 2-photon image belong. This was performed via registration of the Allen Reference Atlas (ARA) (Lein et al., 2007) to the 2-photon data.

The 2011 ARA, a mouse brain atlas which is segmented into 859 total regions, including all cortical regions, was accessed at http://www.3dbar.org/. It is based upon Nissl-staining to delineate the cortical regions. Laminar distribution patterns of local cerebral glucose metabolism have been shown to coincide almost exactly with the regional borders delineated by Nissl staining (Wree et al., 1990). One purpose of comparing vascular architectures is to learn how the vasculature is structured to meet a tissue’s metabolic demands. An atlas whose borders show a good correspondence with metabolic activity (ie. the ARA) was therefore selected. The registration procedure described below was applied to the 2-photon data set in Figure 2.1 (A).

Common tissue landmarks were used to align the ARA to OPT image. The first transformation was rigid (3 translations, 3 rotations) to align the 2 images in the same coordinate system. Application of a thin-plate splines transformation (Bookstein, 1989) allowed for brain distortion caused by handling of the sample and the dehydration procedure.

Identification of common vascular landmarks on large surface vessels (similar to Heinzer et al. (2006)) enabled registration of the OPT to 2-photon data. A rigid body transformation was first applied to the OPT image, followed by a thin-plate splines transformation.

The transformations calculated in registering the ARA to OPT, and OPT to 2-photon, were applied in the same order to the ARA, resulting in its alignment to the 2-photon vascular data, and delineation of the cortical and subcortical regions encompassed by the 2-photon data.
After registration, some of the large surface vessels and sub-surface vasculature were not entirely encompassed by the Atlas (ie.ARA), and were located outside/above the cortical surface of the registered Atlas. This occurred because the thin-plate splines algorithm warps one image to register it to another image, sometimes overcompensating by pushing one part of the cortex in the image inwards (towards its center) too far as a neighbouring region's position is adjusted. If not corrected, these vessels would not be interpreted as belonging to any cortical region. This may affect, for example, calculations of total vascular volume of a cortical region, to which the pial vessels make a significant contribution. To account for vessels located above the cortical surface of the ARA, the cortical surface of the ARA was extrapolated outwards such that all voxels in the 2-photon image were encompassed by the Atlas. These voxels were then assigned a cortical region based on their position in the extrapolated Atlas.

2.3 Establishing the Tools and Analysis Methodologies for Evaluating Vascular Topology

2.3.1 Vessel Tracking

To quantify vessel architecture in the different brain regions, the vasculature must be segmented. This involves tracing out the centres of the vessels and determining their sizes, locations, directions, and the vessels to which each vessel is connected. As the vessels are numerous, an automatic segmentation algorithm (Rennie et al., 2011) was applied to the 2-photon image.

This algorithm finds the centerlines of vessels, and at points along the centerline (termed vertices), calculates the vessel radius, position of the vertex in space relative to a common origin, and direction the vessel is pointing at the vertex. Prior to tracking, the 2-photon image was scaled along the optical axis (z-direction) by a factor of 1.17, producing voxels with dimensions 0.994 x 0.994 x 3.5 µm. This is necessary since in addition to inducing spherical aberrations, the refractive index mismatch between
water \((n_{\text{water}}=1.33)\) and the specimen clearing medium \((n_{\text{BABB}}=1.56)\) results in a scaling of the apparent depth of focus by \(n_{\text{BABB}}/n_{\text{water}}=1.56/1.33=1.17\) for paraxial rays (Tsai et al., 2009). After scaling the image along \(z\), each \(xy\)-plane is convolved with a 2-dimensional Gaussian blurring kernel with a full-width-half-maximum of \(3.5 \, \mu\text{m}\), then resampled to an isotropic voxel size of \(3.5 \times 3.5 \times 3.5 \, \mu\text{m}\).

Tracing of a vessel is initiated by placement of a digital marker near the vessel’s centerline. To decrease the likelihood of any vessel being missed, an algorithm was developed which placed a marker on every voxel in the 2-photon image that possessed a voxel signal greater than or equal to its 6 nearest neighbours. A signal threshold was manually selected by visual examination of the image to exclude placement of seeds on voxels that were deemed to be part of the background noise. Even though multiple seeds may be inside a vessel, this will not cause a vessel to be tracked multiple times: once a vessel has been tracked by a given seed, the other seeds inside this vessel are discarded by the tracking algorithm. Only a minority of seeds in the image are therefore used in the tracking, and ones not used do not contribute to the total time spent by the Vessel Tracking program in segmenting the image. See Figure 2.1 (B) for a picture of these seeds.

A given subimage (field of view about \(0.5 \times 0.5 \, \text{mm}\) in the \(xy\)-plane, about \(1.8 \, \text{mm}\) along the optical axis if using most of the objective’s working distance) in the larger, stitched 2-photon image is typically tracked in about 2-3 days with the seeding algorithm previously described. Since the stitched 2-photon image contains up to 16 of these images, the total processing time would be approximately 1.5 months. Time was reduced by tracking these subimages separately and in parallel. The segmented networks produced by the tracking were merged into a single network (tree) by inferring the connections between vessels intersecting the subimage boundaries. This procedure enabled the entire 2-photon image to be segmented in about 2-3 days. The algorithm finds out the coordinates of the bounding box of each subimage, and in so doing is able to determine the region of overlap between 2 given
subimages. A plane is defined which splits the overlap region in half, and vessels which cross the plane are searched for. Linear interpolation is used to determine the point where a line intersects the plane, and a matching is performed, based on distances between points, where correspondences between points created from segments to the left and right of the plane are found.

The PSF of the 2-photon data is implemented in the vessel tracking code by treating it as a multivariate Gaussian distribution. When tracking a vessel in a given direction, the marginal distribution of the PSF is computed for the plane orthogonal to the vessel axis. This marginal distribution is then convolved with the proposed circular cross-section of the vessel, the latter also approximated as a multivariate (2-D) Gaussian distribution. These convolved distributions define an elliptic contour in the given plane which is tested against the image using the 8 directional gradients in the same manner as for the circular operator described in Rennie et al. (2011). Though the PSF varies linearly with depth below the coverslip for the 2-photon data in this thesis, one PSF for tracking through the entire depth of the image was selected, as a feature taking into account varying PSF with image depth was not yet incorporated into the vessel tracking algorithm. An average of the axial FWHM of the PSF at the coverslip (about 3 µm) and that at about 1.8 mm below the coverslip surface (about 20 µm) yields about 11.5 µm. However, it was found that selection of a single PSF with an axial FWHM of 11.5 µm did not trace the outlines of vessels near the coverslip surface well; as a compromise, an axial PSF of 8 µm was chosen for tracking vessels through the entire cortical depth.

2.3.2 Processing of Vessel Tracking

Ideally, the tracking algorithm will trace the centerlines of only true vessels, and the segmented vascular network will perfectly replicate that of the greyscale image. However, this is not always the case, and the segmentation must be processed accordingly. These processing steps are important for accurately describing the vascular topology.
The tracking algorithm has a variety of criteria to decide when vessel tracking should cease. It is difficult from a user’s perspective to decide on the appropriate stopping threshold. Selection of a high or strict threshold (defined as contrast) results in the program tracing only real vessels but not some of the smaller, weaker signal vessels. These weaker signals tend to emanate from capillaries, as the PSF along the optical axis is typically larger than the capillary diameter (about 6-8 µm). Because capillaries form a significant portion of the cortical vasculature, it is important that they be segmented. The algorithm was therefore selected to run at a lower contrast (i.e., a less strict threshold). Most microvessels are traceable, however the program occasionally does track false vessels. These false vessels are typically in the image background, and are identified by their enlarged diameters relative to neighbouring vessels, and their physiologically unrealistic appearance. Though such vessels represent a minority of segments in the tree (about 5%), their existence distorts blood volume calculations and they must be removed. Large vessels, with their bright signals, are recorded by the algorithm as having high contrast. The false vessels may be identified because they are tracked at a low contrast, yet produce large diameters.

A vessel segment is the portion of a vascular network between two bifurcations, between a bifurcation and a non-connected segment end, or between two non-connected segment ends. In addition to oversized false background vessels, there were a number of vessel segments between a bifurcation and non-connected segment end that pointed vertically straight up or down (parallel to the optical axis). These segments were produced due to the anisotropy of the 2-photon data: the resolution is worse along the optical axis than in the xy-plane, and vessels in the 2-photon data possess elliptic cross-sections, with a larger dimension in the z-direction. Though this property does not affect whether a true vessel centerline is traced, the algorithm sometimes interprets the part of a vessel pointing along z as a separate vessel as well. The vertex at the non-connected end of such a vessel segment was located inside a voxel deemed to be located in the image background based on the signal at that voxel. The vertex at the connected end is located inside the centre of a true vessel. These types of segments,
termed hairs, were excluded from the analysis if their lengths were less than 80 µm. Hairs excluded from the analysis corresponded to about 20% of all segments traced by the tracking. 80 µm was selected as a length ceiling for eliminating hairs as sometimes a very long vessel might be interpreted as a hair if it was not connected to another vessel segment at one of its endpoints. After experimenting with different length ceilings, it was felt that 80 µm eliminated most hairs and prevented elimination of most real vessels.

Figure 2.2 displays an isosurface rendering of the tracked vasculature. Each vessel is approximated as a cylindrical tube, and the diameter of the tube is the diameter as calculated by the tracking algorithm. The isosurface is colour-coded according to cortical region as determined by the registration of the ARA to the 2-photon image. Figure 2.3 shows the location of the strip of 2-photon data relative to the whole brain.
Figure 2.2. Isosurface rendering of the segmented vasculature, colour-coded according to cortical region, as determined by registration to the ARA. The white vessels represent those in the corpus callosum, and the shaded blue vessels below the corpus callosum belong to the subcortical white matter. The 5 regions which will be analyzed are numbered. 1 (orange)=barrel cortex; 2 (red-orange)=somatosensory trunk; 3 (yellow)=posterior parietal association; 4 (red)=primary somatosensory; 5 (purple)=dorsal auditory cortex. Right and left sides of the brain have been marked (R=right, L=left). Scale bar = 1 mm
**Figure 2.3.** This image, viewed from above the cortical surface, shows the cortical surface of the ARA colour-coded according to cortical region. The white box contains the location of the 2-photon image. The angulation of the strip encompassed by the box is advantageous for sampling more cortical regions. Scale bar = 1.5 mm. Arrows indicate the rostral (R) and caudal (CA) directions.
2.3.3 Extracting Penetrating Vessels and Defining the Capillaries

The topological properties of the capillary bed are net-like, while those of the cerebral arterioles and venules are tree-like (Lauwers et al., 2008). Due to these differences, capillaries and non-capillaries are often analyzed separately, and methods must be devised for separating the microvasculature from larger vessels.

A capillary defined in this thesis is both a vessel segment that is not a penetrating vessel or a primary branch off a penetrating vessel, and a segment with a diameter less than 15 µm. A diameter threshold excludes the large diameter surface pial vessels from the analysis, most of which had diameters greater than 40 µm. A threshold of 15 µm was selected based on the inflection point in the histogram of diameter distributions as proposed by Risser et al. (2009).

Penetrating vessels were extracted from the vascular tree by visually examining the tree and placing a marker at the part of a penetrating vessel determined to be closest to the cortical surface. This vessel was traced downwards starting from the marker. At each bifurcation, the tracing algorithm followed the vessel segment which made the smallest angle with the normal to the cortical surface. Normals to the surface were manually defined across the cortex to take into account surface curvature. Theoretically the surface can be separately segmented, a 3D surface fit through the segmented cortical surface, and normals to this 3D surface at various points may be calculated. However, as the cortical surface is relatively flat on about a 0.5-1 mm scale, only 15 normals were required to approximate the cortical surface, which is not a time consuming process. The bifurcation’s position in space determined which normal would be used to select the vessel to follow at the bifurcation. A penetrating vessel would stop being traced once the marker reached the end of a vessel segment that was not connected to any other vessel below it. The centerlines of all penetrating vessels traced with this algorithm are shown in Figure 2.4 (A) below.
Figure 2.4. (A) Centerlines of all penetrating vessels extracted from the segmented vessel network using the algorithm described above. The centerlines are coloured yellow. Scale bar = 1 mm. (B) Separation of capillaries from non-capillaries based on penetrating vessel extraction and diameter cutoff. Non-capillaries are displayed in white. Scale bar = 1 mm.

2.3.4 Calculating Depth Below the Cortical Surface of a Vessel Segment

Since each cortical layer has different metabolic activity levels (Sokoloff et al., 1977), variations in vascular density or volume as a function of cortical depth have been found in rodent somatosensory
cortex (Patel, 1983; Masamoto et al., 2003; Tsai et al., 2009), monkey cortex (Weber et al., 2008), and human cortex (Lauwers et al., 2008). To compute these variations in our segmented networks, a cortical surface must be defined and the depth of every vessel below this surface calculated. In small field of view images, the cortical surface may be approximated by a plane (3D imaging) or a straight line (2D tissue slices) since the cortical surface in a small area is approximately flat. Depth calculations could be performed for each vessel segment in these scenarios by simply finding the distance below the plane (or line) of a vessel segment. In this thesis, the field of view is sufficiently large such that there is significant curvature of the cortical surface (see Figure 2.2). To approximate the cortical surface, for each vessel segment on the cortical surface, the vertex of that segment with the largest z-position value (where vertices closer to the cortical surface have higher z-position values, and the z-axis is parallel to the optical axis) was selected to be a point on the cortical surface. These points are displayed as green markers in Figure 2.5. For each vessel segment in the tree, its depth below the surface is calculated by finding its distance to every marker, and selecting that distance with the minimum value.
Figure 2.5. (A) The centerlines of the entire tracked tree are shown in blue. The green cubes are markers defined to be points on the cortical surface. Approximately 2300 markers are used to define the cortical surface. Scale bar = 1 mm. (B) Magnified view of the inset shown in (A). Scale bar = 0.5 mm.
2.3.5 Extracting Quantitative Parameters

To assess the validity of the imaging methodology, quantitative properties of the vessel network are analyzed, and compared to previously reported values for these same parameters. Five cortical regions in the 2-photon data-set were selected for analysis: barrel field, somatosensory trunk, posterior parietal association, primary somatosensory, and dorsal auditory cortices. These regions were selected because the 2-photon data in these regions comprised at least 20% of the total cortical tissue volume for that region. Anatomical landmarks, such as the superior midline, were used to distinguish between the left and right sides of the brain.

Four metrics were extracted from these regions. These include: capillary length density as a function of cortical depth (mm/mm$^3$) (total length of capillaries per mm$^3$), mean extravascular distance to the nearest vessel, total capillary branch density in each region (number of branches /mm$^3$ of tissue), relative total blood volume (CBV, units of %), and capillary cerebral blood volume (capillary CBV, units of %). Capillary length density as a function of depth is plotted in Figure 2.6, while Table 2.1 contains values for the other parameters. The mean extravascular distance is the distance of each extravascular voxel to the closest blood vessel.

The centre of a vessel segment was determined by averaging the positions of the endpoints of a segment. The cortical region to which a given vessel segment belonged was determined based on the position of the segment centre. Segment lengths were evaluated by summing the distances between successive points in a vessel segment. The number of branches was calculated as the number of bifurcations in the skeletonized network. The CBV in each region was evaluated by determining the total volume of voxels segmented from the vascular network over the total volume of the cortical region encompassed by the 2-photon data.
Figure 2.6. Plot of capillary length density versus cortical depth.
Table 2.1. Morphometric parameters according to cortical region as defined by registration to the ARA. Error estimates in the values were obtained by examining the variations in these parameters between the left and right side of the brain for regions close to the midline. These regions were the Secondary Motor cortex (green, Figure 2.2 A), and the Ventral Retrosplenial cortex (yellow region between the left and right Secondary Motor Cortices, Figure 2.2 A).

<table>
<thead>
<tr>
<th>Region</th>
<th>Capillary branch density (#/mm³)</th>
<th>Capillary CBV (%)</th>
<th>Total CBV (%)</th>
<th>% Blood Volume Contribution from large vessels</th>
<th>Mean Extravascular Distance (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barrel (right)</td>
<td>12 300 ± 2400</td>
<td>4.0 ± 0.8</td>
<td>7.7 ± 1.5</td>
<td>47 ± 9</td>
<td>18.1 ± 3.6</td>
</tr>
<tr>
<td>Somatosensory Trunk (right)</td>
<td>8000 ± 1600</td>
<td>2.8 ± 0.6</td>
<td>5.2 ± 1.0</td>
<td>46 ± 9</td>
<td>21.0 ± 4.2</td>
</tr>
<tr>
<td>Posterior Parietal (left)</td>
<td>8000 ± 1600</td>
<td>3.2 ± 0.6</td>
<td>6.5 ± 1.3</td>
<td>51 ± 10</td>
<td>19.5 ± 3.9</td>
</tr>
<tr>
<td>Primary Somatosensory (left)</td>
<td>5500 ± 1100</td>
<td>2.6 ± 0.5</td>
<td>4.1 ± 0.8</td>
<td>37 ± 7</td>
<td>22.9 ± 4.6</td>
</tr>
<tr>
<td>Dorsal Auditory (left)</td>
<td>6800 ± 1400</td>
<td>3.2 ± 0.6</td>
<td>7.6 ± 1.5</td>
<td>58 ± 12</td>
<td>21.1 ± 4.2</td>
</tr>
</tbody>
</table>

2.4 Summary of the Results Obtained with the Imaging Methodology

The tissue transparency induced via optical clearing in BABB enabled imaging through the entire cortical depth of the intact mouse brain, as demonstrated in Figure 2.1 (A). Pial vessels are clearly visible, as well as penetrating vessels and their branches, microvessels, and white matter vessels.
Figure 2.3 depicts the location of the vessel network in Figure 2.2 relative to the whole brain. The brain was imaged at an angle to a true coronal section to gather information on more cortical areas. This accounts for the lack of symmetry in the colour-coding of Figure 2.2.

The capillary length density versus cortical depth is plotted in Figure 2.6. Peak length density values range from about 400-800 mm/mm$^3$, with the barrel cortex containing the highest vascular length density. For each region, the length density increases moving deeper into the cortex, until a peak length density is reached and then decreases as the white matter is approached.

Table 2.1 lists the capillary CBV, total CBV, and branch density for the 5 regions. Similar to the length density measurements, the highest capillary and total CBV, and branch density values are found in the barrel cortex. The lowest values are found in the primary somatosensory area. The contribution of non-capillaries to the total vascular volume ranged from 37-58 %, even though they are fewer than 25% of all vessel segments.
Chapter 3

3.1 Advantages of the Imaging Methodology

The purpose of this thesis was to develop a novel imaging methodology for assessing regional differences in the angioarchitecture of the mouse brain. The methodology combines 2-photon microscopy with optical clearing to image through the whole cortical depth of an unsectioned mouse brain. Imaging stitching is incorporated into the procedure to increase the effective field of view of the microscope images, while maintaining the same resolution. Registration of the ARA to the 2-photon data via an intermediate OPT image enabled delineation of the cortical regions in the data set. Automatic vessel segmentation techniques applied to the data allowed quantitative parameters to be analyzed and compared between regions.

An advantage to the methodology is that the brain remains unsectioned during the procedure. This prevents distortion of the vasculature at the edges where the brain is cut, and is particularly relevant for thinner sections since proportionately more blood vessels in a thinner section will be distorted. As depicted in Figure 2.6, imaging through the whole cortical depth of the intact mouse brain and well into the white matter was achieved. The methodology enables capillary visualization over large volumes without destroying tissue, and the size of the data is only limited by the imaging time or computer storage space available. Localization of the 2-photon data was achieved via digital registration of the ARA to the data, whereas excised tissue is challenging to localize (Heinzer et al., 2006).

A further advantage of our methodology is ease of use and speed. The sample is dehydrated and cleared in about 3-4 days. Another well-known clearing agent, the urea-based ScaleA2 (Hama et al., 2011), requires incubation of a mouse brain for a period of at least 2 weeks. At the end of this period, the transparency is still not of the quality as BABB (experiments conducted separately by author of this
ScaleA2-cleared brains are more fragile than those immersed in BABB (Hama et al., 2011; experiments conducted separately by author of this thesis); a variant on ScaleA2, ScaleU2, renders brains less fragile but the clearing time is on the order of several months (Hama et al., 2011). The glass well in which our mouse brain is held during imaging may be easily acquired; an off-the-shelf glass container about the same size as a mouse brain will suffice. Each single block in the overall image mosaic may be produced in about 11 minutes, which enables acquisition of large data sets in a short time frame. This is advantageous for high throughput imaging. The vessel segmentation algorithm was able to segment out the vasculature in our stitched 2-photon images in less than three days. In a total time of about 2 weeks, beginning with when the mouse is first perfused, a large, high-resolution data set may be obtained with tens of thousands of vessels segmented.

Other techniques can image a whole mouse brain (including vasculature) at approximately 1 µm resolution. Some of these include micro-optical sectioning tomography (MOST) (Li et al., 2010), all-optical histology with ultrashort laser pulses, and serial two-photon tomography (STP tomography) (Arganda-Carreras et al., 2012). They are able to image through the entire brain depth via sectioning the tissue, either with a special microtome (MOST and STP tomography), or with ultrashort laser pulses (All-Optical histology). Because the tissue is destroyed, it can not be imaged again at a later date. The brains in this thesis are not destroyed and may be stored indefinitely in BABB. They may be rehydrated and stained for cytoarchitecture or other features of interest, allowing cellular data to be incorporated with the vascular images. Whole-brain, high resolution imaging techniques require specialized equipment available to few labs. Purchase of the appropriate equipment is expensive, and proper operation of the machinery may depend upon extensive computer programming knowledge. Our imaging methodology merely requires access to a standard 2-photon microscope. These are accessible at many major universities and/or research institutes. The stitching program, XuvTools, may be downloaded free online.
Several novel image analysis algorithms were developed. The tracking algorithm applied in this thesis has been previously used (Rennie et al., 2011), where tracking of a placental tree imaged with micro-CT was initiated by manually placing a single seed in an artery. Vessel branches missed in the first round of tracking were segmented in a second round initiated by placing seeds on untracked branches. This method of seed placement would not be effective for the 2-photon images generated in this thesis as thousands of vessels remained untracked after the 1\textsuperscript{st} round; hence a seeding algorithm was developed to automatically place seeds on every vessel based on local signal maxima inside vessels. Further, imperfections in perfusions may cause breaks in vessels and isolated vessel segments in images. These would remain untracked in our images had a novel seeding algorithm not been developed. Since the field of view is only approximately 0.5 mm in the anterior-posterior direction, a number of vessels enter and exit the field of view. This results in separate connected vessel networks in the image which may be tracked as the result of this seeding algorithm. Investigators that have used 3D segmentation techniques to study capillary networks have not encountered this difficulty (Cassot et al., 2006; Lauwers et al., 2008; Risser et al., 2009; Tsai et al., 2009). Their grayscale vascular images are binarized via selection of a signal threshold to separate the vasculature from the background, and these binary images are eroded until only the centerlines of vessels remained. This results in the segmentation of all vessels regardless of how many separated separate connected networks exist by selection of a binary threshold. However, the drawback to binarization techniques are that they are highly sensitive to topological inconsistencies introduced by noise. Moreover, it would not be possible to incorporate an explicit model of the PSF into a binarization/skeletonization process, thereby exacerbating the hair problem. Skeletonization procedures are prone to hairs as noisy boundaries of the vessel surface are often transformed into short free-ends (Tsai et al., 2009).

The nature of our tracking algorithm necessitated development of unique methods for processing the segmented vascular network. Use of lower stopping thresholds enabled tracking of smaller, weaker
signal vessels. The cost of using a lower threshold was the tracing of false, over-sized vessels in the image background. These vessels were automatically identified and eliminated, allowing for segmentation of the majority of capillaries, while maintaining realistic blood volume measurements. Elimination of vertical hairs enhanced the geometrical realism of the tracked vessel network. Each hair emanates from a branch point, and eliminating these hairs provides accurate measurements of parameters such as branch density.

Our algorithm for extracting penetrating vessels yielded a more accurate definition of a capillary network. Diameter thresholds are often selected in the literature, however the threshold to use is not universal. It depends on imaging modality, specimen preparation, species, and vessel segmentation techniques, each of which may impact measured vessel diameters. Penetrating vessels may be similar in diameter to capillaries (Blinder et al., 2013; personal observation), and a size threshold may not eliminate all penetrating vessels. By separately segmenting these vessels with semi-automatic methods, and excluding the pial vasculature with a diameter threshold, a less arbitrary definition of a capillary bed is obtained. Approximately 300 penetrating vessels were extracted using our algorithm for tracing the penetrating vessels. The manual identification and placement of seeds consumed a few hours; these vessels were automatically traced in minutes. Manually tracing each of these penetrating vessels, and deciding by eye which branch to follow at each bifurcation, would have consumed unnecessary additional hours.

Our approach for defining the cortical surface and calculating cortical depth is fully automated, and is effective for any degree of surface curvature. Manual approaches may be used such as dividing the skeletonized network into sufficiently small subregions to minimize surface curvature in each subregion. In each subregion, the surface may be approximated with a plane, and the cortical depth of a vessel
segment calculated by finding its distance to the plane. This approach is more labour-intensive than ours and provides a coarser approximation of the cortical surface.

The motivation for this thesis was to develop an imaging methodology suitable for imaging large portions of the cortical microvasculature in three-dimensions. Analysis of the cortical networks would hopefully shed light on the organization of the cortical vasculature, and how vessel networks are arranged to meet a tissue’s metabolic demands. As a first step, large portions of the vasculature of different cortical regions were imaged, and networks were segmented. For a preliminary analysis, capillary length density, branch density, and capillary and total CBV were calculated and compared to commonly reported values in the literature. Similarities between values obtained with this methodology and published values suggest that our technique is well-suited to analysis of the cortical vasculature.

The length density values (Figure 2.6) are within the range published for various species. Peak values range from 400-800 mm/mm$^3$. In volumetric data from human cortex, Lauwers et al. (2008) obtained values ranging from 400-600 mm/mm$^3$. In volumetric data from mouse cortex, Tsai et al. (2009) found an average density of 880 ± 170 mm/mm$^3$ which included one volume from frontal cortex and three from parietal cortex (one of which was from the barrel area). Boero et al. (1999) calculated length densities from 700-1200 mm/mm$^3$ from manual traces of thin tissue sections for the somatosensory and auditory areas. Though these values appear high, in tissue sections from layers 2/3 of the hindlimb representation of rat motor cortex, Tata and Anderson (2002) found an average capillary length density of 215 mm/mm$^3$. These variations may be explained by discrepancies between cortical areas, methods, and specimens (Lauwers et al., 2008). Nevertheless, our range of 400-800 mm/mm$^3$ is comparable to other investigators. The qualitative shapes of our curves are in agreement with Masamoto et al. (2003), who in studies of the rat somatosensory cortex, found lower densities near the cortical surface, highest densities in the middle layers of the cortex, and a steady decrease in density as the white matter was
approached. The middle of the cortex includes layer 4, which is the target of thalamo-cortical afferents in sensory cortex and has been shown to possess the highest level of metabolic activity in the cortex (Hevner et al., 1995). The higher capillary densities in these areas suggest a close connection between vessel distribution and functional activities. There is a possibility that the higher densities in our data encompass layer 4 entirely and overlap adjacent layers which may have strong synaptic connections with layer 4 (Masomoto et al., 2003). This correlation requires further investigation for confirmation, including a description of the cytoarchitecture of the areas studied.

Heinzer et al. (2008) reported branch density values ranging from 6000-10000 (#/mm³) in 1 mm³ samples of parietal cortex from three different mice. A branch density of about 8000 (#/mm³) was found in posterior parietal association cortex, correlating well with Heinzer et al. (2008). Chugh et al. (2009) found a mean total CBV of 7.9±0.7 % in mouse cerebral cortex, and our parameters ranged from 4-8 % depending on cortical area. Though less than 25% of all vessel segments, non-capillaries contributed about 40-60 % of the total blood volume due to their large diameters. Chinta et al. (2012) found capillaries to comprise 77% of all blood vessel segments in primary somatosensory rat cortex. Lauwers et al. (2008) reported similar capillary blood volume contributions in human cortex (hovering around 50% of total blood volume). Risser et al. (2009) calculated an average of about 60% of blood volume contribution from capillaries in Rhesus Macaque cortex.

Values for the mean extravascular distance ranged from about 18-23 µm. From in vivo images of rat primary somatosensory cortex (forelimb representation), Chinta et al. (2012) calculated a mean extravascular distance of approximately 25 µm, very similar to the results reported here.

Parameters calculated in this thesis are in agreement with those obtained by other investigators, indicating robust vessel quantification. The technique developed is well-suited to comparing vascular architectures across the mouse cortex. This thesis is a first step towards making this comparison.
3.2 Limitations of the Imaging Methodology

Use of BABB as a clearing agent results in high specimen transparency, however its chemical and optical properties limit the scope of our technique. These limitations include inability to access all cortical regions, difficulties visualizing the cellular architecture of the brains, and optical aberrations in the 2-photon images. This section discusses each of these limitations, and methods for correcting them.

(1) Inability to access all cortical regions

BABB has corrosive properties and dissolves plastics. The microscope objective used in this thesis is designed to be immersed in water, and may be damaged if it contacts BABB. To separate the BABB-cleared brain from the objective, it was placed in a well filled with BABB. This well was then sealed with a glass coverslip. By dipping the tip of the objective in a water drop placed on the coverslip, the objective was employed in its natural environment, while allowing the sample to be immersed in BABB.

Though effective for imaging brain regions closer to the midline, regions closer to the sides where cortical surface curvature was more pronounced were difficult to access. This difficulty arises from the working distance of the objective (2 mm). The height of the well is chosen such that when the coverslip is placed over the well, the superior sagittal sinus (brain midline) is just below the coverslip (anywhere from 0-100 µm below). Moving laterally across the cortex beginning from the midline, the distance of the cortical surface to the coverslip increases due to brain curvature. For regions with surfaces located over 1 mm below the coverslip, imaging through the entire cortical depth, or even accessing these regions, with a 2 mm working distance objective was difficult or impossible.

This problem may be resolved by installation of a brain rotator inside the well, or a moveable platform. Cortical areas previously inaccessible, such as the entorhinal and perihinal cortex, might then be accessible. These regions, which are involved in the brain’s memory system, are affected by Alzheimer’s
Juottonen et al., 1998). Alzheimer’s may potentially be classified as a vascular disorder (de la Torre, 2002), and vascular information pertaining to the entorhinal and perihinal cortices’ vasculature may prove useful in understanding the disease.

(2) Visualizing brain cellular architecture

All cortical neurons depend upon the vasculature to perform their tasks, and the interactions between neurons and blood vessels are constrained by the architecture of the underlying vascular network (Kleinfeld et al., 2011). The density and architecture of the vasculature relative to the neurons is important for understanding the spatial localization of brain-blood cell interactions (Tsai et al., 2009), and bears directly on the limit of cortical function and nervous system resource management. In this thesis, the 2-photon and OPT images contain strictly vascular information, and no tissue information. Gleave et al. (2012) showed that certain somatosensory regions, visual cortex, and auditory cortex could be uniquely identified with autofluorescence imaging. Neither OPT nor the 2-photon images in Chapter 2 possessed these autofluorescent signatures. The fluorescent contrast agent perfused through the vasculature, Nile Red, was selected due to its intense fluorescence in organic solvents (Greenspan et al., 1985), which enabled the visualization of smaller vessels deeper in tissue. It was strongly fluorescent in the green (autofluorescent) channels, forcing a low camera exposure time for OPT imaging in the autofluorescent channels. This was implemented in order that light emitted from the larger vessels would not saturate the camera and produce artifacts in the OPT reconstruction. The low camera exposure resulted in a minimal autofluorescence detection, and unique tissue signatures could not be discerned. In future, contrast agents with a smaller band-width than Nile Red might be used. An example is the Texas Red BODIPY dye offered by Life Technologies (Product D-6116). BODIPY dyes contain carbon rings and a boron atom as part of their core structure. They are soluble in Mercox (the
perfusion material), and the Texas Red fluorophore conjugated to them demonstrates weak fluorescence below 600 nm, in contrast to Nile Red (Greenspan et al., 1985).

Relating cellular and vascular architecture is more easily accomplished with use of optical clearing procedures more compatible with fluorescent proteins. Fluorescent protein expressing mice enable visualization of different neuronal types, along with their axons and dendrites (Livet et al., 2007; Feng et al., 2000). BABB quenches fluorescent protein expression (Becker et al., 2012), so these mice are not compatible with the optical clearing described in this thesis. Becker et al. (2012) overcame this difficulty by dehydrating mouse brains in tetrahydrofuran (THF) instead of methanol, and clearing in dibenzyl ether (DBE) instead of BABB. This method preserved GFP fluorescence, and because DBE has approximately the same refractive index as BABB, has similar clearing capabilities. Future research may incorporate THF/DBE clearing and fluorescent protein-expressing mice. Images of cells may be segmented, similar to what is done for the vasculature. Cellular and vascular architectures may be simultaneously analyzed.

Because CLARITY (Chung et al., 2013) does not require dehydration and organic solvents to render tissue transparent, it is compatible with fluorescent proteins and cell staining. An additional advantage of CLARITY over BABB is that it renders both the cortex and subcortical white matter transparent, increasing the number of brain structures that may be analyzed. If a sample is cleared with CLARITY, as opposed to through BABB immersion, the vasculature and cellular architecture of the entire brain may be visualized. The same tools and techniques developed in this thesis for imaging and analyzing BABB-cleared brains could be used to image CLARITY-cleared mouse brains, with minimal adaptations. Analyzing white matter vasculature in parallel with cortical vasculature may be of interest as axons of cortical neurons sometimes connect with subcortical structures. As a result of these connections, cortical physiology and metabolism is interrelated with that of subcortical structures. One reason for
analyzing the cortical vasculature is to better understand how vascular networks are patterned to meet a cortical region’s metabolic demands. As these metabolic demands are tied in to those of the white matter, analysis of white matter vasculature may aid in interpreting the patterning of the cortical vasculature. A further advantage of CLARITY is that the refractive index of FocusClear, 1.45, is closer to that of water than BABB (1.56). This decreases the effects of spherical aberrations on image quality (see below).

(3) Optical aberrations

The refractive index mismatch between the specimen immersion medium (BABB, 1.56) and the objective immersion medium (water, 1.33) causes spherical aberrations and distortions in the image (see Introduction). Some distortions, such as the “flares” emanating from vessels, are removed with the deconvolution software used in this thesis. Resolution along the optical axis decreases linearly from a full-width-half-maximum 3 µm at the cortical surface to about 20 µm (unpublished data) at 2 mm depth. Since microvessels are typically less than 10 µm in diameter, those more than 1 mm below the coverslip surface emit weaker signals during imaging since their diameter is less than the focal spread of the objective. These vessels are sometimes missed by Vessel Tracking due to their low contrast. Scale (Hama et al., 2011), a urea-based tissue clearing reagent, may overcome this difficulty since it possesses a similar refractive index to water, minimizing spherical aberrations. Since BABB’s refractive index is closer to that of tissue proteins, it renders samples more transparent. There is a trade-off between transparency and refractive index. We opted for higher transparency to ensure deep tissue imaging. One possibility to guarantee tissue clarity and no index mismatches is to image with a BABB-immersion objective. Leica Inc. produces such objectives. These are not necessarily compatible with the Olympus 2-photon microscopes due to the different microscope designs of the two companies. Potential issues are whether the back aperture of the Leica objective may be completely filled, leading to a decreased
resolution; and reduced quality in lateral colour correction and astigmatism (email communication by Dr. Andrea Pfeiffer, Leica Inc. representative). This avenue remains to be investigated.

3.3 Future Research and Applications of the Technique

The imaging technique developed for this M.Sc. thesis is a first step towards a quantitative analysis of the cortical microvasculature. The analysis presented here demonstrates the potential of the technique by assessing several commonly reported parameters. Additional parameters could be assessed to address specific hypotheses regarding vascular topology, such as whether it varies across the cortex. Examples include inter-capillary distances, and the penetrating arteriole to venule ratio. Capillaries are often spaced less than 30 µm apart in the mouse brain (Meyer et al., 2008), resulting in short oxygen diffusion distances. Variability in the intercapillary distance may provide insight into oxygen exchange with tissue, and signify regional, microscale vulnerability to hypoperfusion. Nguyen et al. (2011) found that differences in nearest-neighbour spacing between penetrating venules and arterioles, combined with the capillary topology, predict the severity and spatial extent of blood flow reduction after occlusion of either vessel type. Differentiating between arterioles and venules may shed light on the relationship between stroke location in the vascular network and its severity, and could aid in elucidating the vulnerability of different brain regions to vessel occlusions.

Future research will take advantage of the three-dimensional nature of the images to extract more 3D metrics. An example of such a metric is a calculation of the shortest path distances to the nearest penetrating arterioles. This could illustrate the degree of overlap of each penetrating arteriole irrigation volume. Blood flow modeling may be incorporated to determine how network structure impacts nutrient distribution to tissues. Modeling is performed on the segmented vascular tree. To accurately model the flow, the graph should be free of spurious connections and erroneous gaps (Kaufhold et al., 2012). Though many false vessel segments were removed in this thesis, some false gaps in the network
likely remain. Local regions with low signal to noise ratio, incomplete vessel perfusions, and intrinsic contrast gradients along the length of a vessel affect the perceived connectivity of the structure in the grayscale data. Local contrast gradients may stem from inhomogeneous contrast agent distribution along a vessel, variations in size of a vessel along its length, and the vessel being partly shadowed by a larger vessel above it closer to the coverslip. This will result in some vessel segments being disconnected in the segmented network. Risser et al. (2008) applied tensor voting to connect vessel segments, but relied on the segmented graph of the vasculature and not the underlying greyscale data for gap filling. Kaufhold et al. (2012) applied threshold relaxation to correct connectivity defects, where a binary image of the grey scale image is produced with the threshold lowered until 2 endpoints in the vascular graph are connected by the greyscale data. A number of metrics such as bridging strand tortuosity, length, and distance profile to nearest vessel, were considered in selecting the optimal strand for connecting 2 vessel endpoints in the graph. Looking ahead, incorporating similar algorithms (tensor voting or threshold relaxation) into the graph processing may improve analysis results, which could be validated by comparing flow simulations with and without such algorithms.

The methodology was employed to image approximately 0.5 mm thin strips in the brain. This may be extended to obtain vascular images of the entire neocortex. This would require many imaging hours. Incorporation of an automatic, computer controlled moveable stage may hasten this process. Segmentation of the network would result in a map of the entire cortical vasculature, termed an angiome (Kleinfeld et al., 2011). The angiome would be amenable to blood flow modeling. Vascular variations within brain regions might be examined. If cell staining techniques are incorporated into the imaging procedure, the relation between the vasculature and cell type and location may be investigated. By using the angiome to characterize the vasculature of healthy, normal brain regions, abnormal brain states, such as in stroke or Alzheimer’s, could be identified based on vascular oddities. Since vascular changes are known to occur early in Alzheimer’s disease pathogenesis, the angiome may be a starting
point to aid in disease diagnosis. A map of blood vessel patterning and its relationship to blood flow may elucidate how damage to a specific vessel impacts brain health.

Though the degree to which the vascular architecture varies across the brain remains to be investigated, the techniques and tools developed here provide the basis for examining this variation. This thesis is the first step in an imaging journey to clarify how vascular networks are patterned to meet a tissue’s metabolic demands.
References


Lindvere L. “Functional Stimulation Induced Change in Cerebral Blood Volume”. Master’s Thesis, University of Toronto, Department of Medical Biophysics (2011).


