Functional Stimulation Induced Change in Cerebral Blood Volume:

A Two Photon Fluorescence Microscopy Map of the 3D Microvascular Network Response

Liis Lindvere

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Medical Biophysics
University of Toronto

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ABSTRACT
The current work investigated the stimulation induced spatial response of the cerebral microvascular network by reconstruction of the 3D microvascular morphology from in vivo two photon fluorescence microscopy (2PFM) volumes using an automated, model based tracking algorithm. In vivo 2PFM imaging of the vasculature in the forelimb representation of the primary somatosensory cortex of alpha-chloralose anesthetized rats was achieved via implantation of a closed cranial window, and intravascular injection of fluorescent dextran. The dilatory and constrictory responses of the cerebral microvascular network to functional stimulation were heterogeneous and depended on resting vascular radius and response latency. Capillaries experienced large relative dilations and constrictions, but the larger vessel absolute volume changes dominated the overall network cerebral blood volume change.
# TABLE OF CONTENTS

ABSTRACT ............................................................................................................................. ii

TABLE OF FIGURES ................................................................................................................ v

GLOSSARY OF TERMS ............................................................................................................. vii

1.0 INTRODUCTION / BACKGROUND ................................................................................ 1

1.1 Neurovascular Coupling ................................................................................................. 1

1.2 Objective ......................................................................................................................... 4

1.3 Hypothesis ......................................................................................................................... 4

1.4 Somatosensory Pathway ................................................................................................. 4

1.5 Light Interaction with Tissue .......................................................................................... 5

1.6 Two Photon Fluorescence Microscopy (2PFM) ............................................................... 7

2.0 METHODS ......................................................................................................................... 14

2.1 Animal Model .................................................................................................................. 14

2.2 Two Photon Fluorescence Microscopy (2PFM) ............................................................. 17

2.3 Point Spread Function (PSF) ........................................................................................ 18

2.4 Image Acquisition .......................................................................................................... 19

2.5 Analysis ......................................................................................................................... 22

3.0 RESULTS ........................................................................................................................... 30

3.1 Anatomical Data ............................................................................................................. 30

3.1.1 Characteristic Attenuation Length (CAL) ................................................................. 30

3.1.2 Total Cerebral Blood Volume (CBV) ......................................................................... 31

3.2 Functional Data .............................................................................................................. 31

3.2.1 Paradigm 1 ............................................................................................................... 32

3.2.2 Paradigm 2 ............................................................................................................... 33

3.2.2.1 Temporal Response of Paradigm 2 ...................................................................... 34

3.2.3 Paradigm 3 ............................................................................................................... 38

3.2.3.1 Temporal Response of Paradigm 3 ...................................................................... 38

3.3 Map of Microvascular Response to Stimulation ............................................................. 42

3.4 Conclusion ...................................................................................................................... 42
4.0 DISCUSSION AND FUTURE WORK ................................................................. 43
  4.1 Discussion .............................................................................................. 43
  4.2 Summary of Major Findings ..................................................................... 49
  4.3 Future Work ............................................................................................ 49
REFERENCES .................................................................................................. 51
APPENDIX ....................................................................................................... 55
TABLE OF FIGURES

Figure 1: Absorption coefficient (probability of photon absorption in tissue per unit path length) of prominent tissue components within the ‘optical window’. With 810 nm excitation wavelength oxy (O2Hb) and deoxy (HHb) hemoglobin are the dominant absorbers (reproduced from www.bme.arizona.edu). .................................................................................................................. 6

Figure 2: The light path of an example two photon (pink underlay) microscope includes several dichroic mirrors (RDM690, ADM720, MV/G, MC/Y, MG/R, MDM680), filters (LSM/ R690/ UWU2/ U-MNB2/ U-MWG2), galvanometer controlled mirrors, prisms, and external PMTs (BXD1, BXD2). .................................................................................................................. 12

Figure 3: A: Closed cranial window preparation with a dental cement well to hold water for water immersion objective. B: Open cranial window prior to closing with coverslip. ............ 17

Figure 4: Imaging of fluorescently labeled plasma is achieved after implantation of a closed cranial window. The well serves as a basin for water to immerse the high NA (1.05) 25 X objective for two-photon fluorescence microscope imaging (2PFM). ................................................................. 18

Figure 5: The intensity profiles (lateral x; and axial z) of a sub-resolution (0.17 µm diameter) fluorescent bead acquired with 25 X magnification and 1.05 NA. The full width at half max (FWHM) is estimated (by Gaussian fit - red) to be approximately 0.33 µm in x, and 1.36 µm in z). .................................................................................................................................................... 19

Figure 6: 2D planar data is acquired over time in sequential depth (100 - 400 µm), then reconstructed to volumes with spatial resolution of 1.6 x 1.6 x 3 µm and effective temporal resolution of 0.786 sec/volume. Stimulus in paradigm 2 is applied during frames 3, 4, 5 and 24, 25, 26 (paradigm repetition not shown). ........................................................................................................................................ 22

Figure 7: Data processing pipeline. Functional volumes are subjected to motion correction, Gaussian filtering, and intensity normalization across cortical depths prior to location, tangent, and radius estimating algorithm. General linear model analysis is done with AFNI (Cox, 1996). ........................................................................................................................................ 27

Figure 8: A: 3D rendering of 2PFM image of cortical microvasculature in forelimb representation of the primary somatosensory cortex B: Segmentation of 2PFM image done with commercially available software, Imaris (Bitplane). ........................................................................................................ 28

Figure 9: View of the plane orthogonal to the longitudinal vessel axis. A: initial guess based on Imaris segmentation of the anatomical acquisition (figure 8b). B: final estimate using in-house multi-scale algorithm allowing small adjustments to centre line location and radius value........ 28

Figure 10: Radius and position estimation from one time point using in-house algorithm segmentation (red) overlaid on temporal mean of functional data. Left: x-y slice (parallel to cortical surface) approximately 150 µm below superficial pial layer. Right: x-z (top) and y-z (bottom) slice. Profile of cortical vasculature between 100 to 400 µm includes many penetrating vessels. ........................................................................................................................................ 29
Figure 11: The characteristic attenuation length (CAL) curve of an example data set. CAL is defined as the depth at which there is an e-2 fold decay in normalized fluorescent signal of the brightest 10% of pixels as described in (Kobat et al, 2009). CAL is estimated at 375 μm for this data set. ..................................................................................................................................................30

Figure 12: Distribution of vessels from 0 to 600 μm data set (n = 2) (left). Major pial vessels were avoided. Imaging the full available depth (0 to 600 μm), majority of dilations and constrictions were seen between 100 to 400 μm (right), motivating further scrutiny of this depth of interest. ........................................................................................................................................33

Figure 13: Distribution of vessels in sub - volume (cortical depth from 100 to 400 μm) (n = 7). ..........................................................................................................................................................................................................................................................34

Figure 14: A, B, C: Average vertex-wise timecourse of paradigm 2 across subjects for dilations (red), constrictions (blue) and modeled HRF (black). Stimulation presentation is shown in yellow. The mean is shown in bold with the inter-subject standard deviation shaded for quick, medium, and slow responding vertices. D, E, F: Gamma variate fit to average timecourses for quick, medium, and slow responding vertices. Active vertices were first averaged within each subject, then averaged across subjects (n = 7), and finally averaged across the two presentations of the stimulus. ..................................................................................................................................................37

Figure 15: A, B: Average vertex-wise time course of paradigm 3 across subjects for dilations (red), constrictions (blue) and modeled HRF (black). Stimulus presentation is shown in yellow. The mean is shown in bold with the inter-subject standard deviation shaded. C, D: Gamma variate fit to average time courses for quick and mid-latency responding vertices. Active vertices were first averaged within each subject, then averaged across subjects (n = 5 for quick HRF; n = 7 for mid-latency HRF), and finally averaged across the five presentations of the stimulus..................................................................................................................................................40

Figure 16: Map of estimated stimulation-induced change in radius (Δr) for paradigm 2, dilations (red) and constrictions (blue) of a single subject for quick, medium, and slow latency responses (left). Regions of statistically significant correlation after clustering (right) are shown for each HRF response. Scale bar in green (bottom right) is 100 μm. ........................................................................................................................................41
## GLOSSARY OF TERMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>3D</td>
<td>three dimensional (spatial)</td>
</tr>
<tr>
<td>AFNI</td>
<td>Analysis of Functional Neuro Images</td>
</tr>
<tr>
<td>AOM</td>
<td>acousto-optic modulator</td>
</tr>
<tr>
<td>BOLD fMRI</td>
<td>blood oxygen level dependent functional magnetic resonance imaging</td>
</tr>
<tr>
<td>CAL</td>
<td>characteristic attenuation length</td>
</tr>
<tr>
<td>CBV</td>
<td>cerebral blood volume</td>
</tr>
<tr>
<td>DOT</td>
<td>diffuse optical tomography</td>
</tr>
<tr>
<td>fIO₂</td>
<td>fraction of inspired oxygen</td>
</tr>
<tr>
<td>FOV</td>
<td>field of view</td>
</tr>
<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
</tr>
<tr>
<td>GLM</td>
<td>general linearized model</td>
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<td>GVD</td>
<td>group velocity dispersion</td>
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<tr>
<td>HRF</td>
<td>hemodynamic response function</td>
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<tr>
<td>IM</td>
<td>intramuscular</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
</tr>
<tr>
<td>NIRS</td>
<td>Near Infrared Spectroscopy</td>
</tr>
<tr>
<td>NVU</td>
<td>neurovascular unit</td>
</tr>
<tr>
<td>O₂Hb/HHb</td>
<td>oxyhemoglobin / deoxyhemoglobin</td>
</tr>
<tr>
<td>OT</td>
<td>onset time</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PMT</td>
<td>photon multiplier tube</td>
</tr>
<tr>
<td>PSF</td>
<td>point spread function</td>
</tr>
<tr>
<td>S1FL</td>
<td>forelimb representation in the primary somatosensory cortex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>SNR</td>
<td>signal to noise ratio</td>
</tr>
<tr>
<td>TTP</td>
<td>time to peak</td>
</tr>
<tr>
<td>2PFM</td>
<td>two photon fluorescence microscopy</td>
</tr>
<tr>
<td>VPL</td>
<td>ventro-posterolateral</td>
</tr>
<tr>
<td>WD</td>
<td>working distance</td>
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</table>

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
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<tr>
<td>$\Delta r$</td>
<td>change in radius (delta r)</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>wavelength (lambda)</td>
</tr>
<tr>
<td>$\lambda_{ex}/\lambda_{em}$</td>
<td>excitation/emission wavelength (lambda)</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>standard deviation (sigma)</td>
</tr>
<tr>
<td>$p_0$</td>
<td>average laser intensity</td>
</tr>
<tr>
<td>$\delta$</td>
<td>2-photon cross section</td>
</tr>
<tr>
<td>$\tau_p$</td>
<td>pulse duration</td>
</tr>
<tr>
<td>$f_p$</td>
<td>repetition rate of laser</td>
</tr>
<tr>
<td>$\eta$</td>
<td>refractive index</td>
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1.0 INTRODUCTION / BACKGROUND

The study of brain development, its function, and its compromise in the course of neurodegeneration can profoundly benefit from quantitative data on microvascular architecture and hemodynamics. Healthy functioning of the brain is critically dependent on continuous cerebral blood flow for the supply of metabolites, cooling, and removal of catabolic products. The relationship between local neuronal activity and subsequent changes in cerebral blood flow, termed neurovascular coupling, occurs on the level of capillaries. Moreover, most neurodegenerative and cerebrovascular diseases are associated with a disturbance in neurovascular coupling (D'Esposito et al, 2003).

1.1 Neurovascular Coupling

The neurovascular unit (NVU) is a dynamic structure composed of the capillary's glycocalyx, endothelium, and basement membrane, pericytes, astrocytic end feet, and neurons (Abbott et al, 2010). The exchange of oxygen from microvasculature to parenchyma is one function of the NVU. NVU also gives rise to the blood-brain barrier (BBB) and neuroimmune interface. Neuronal activity is reflected in the number of neurons firing an action potential and/or the frequency with which they fire and comprises a complex sequence of cellular, metabolic, and vascular processes that occur on the level of the NVU. Neuronal activation leads to increased consumption of oxygen and glucose. Moreover, neuronal activation is associated with the generation of vasoactive substances that act on the neighbouring vessels inducing changes in smooth muscle tone and vascular diameter. The caliber adjustments in proximal microvasculature are heterogeneous across different regions of the brain (Harrison et al, 2002), among cortical layers (Silva & Koretsky, 2002), and axially along capillaries (Peppiatt et al, 2006),
(Fernández-Klett et al, 2010). The mechanisms behind neurovascular coupling are still being researched; however, it is generally accepted that neurovascular coupling is a feedforward process: neurons either communicate directly to the proximal vasculature or act on astrocytes to release vasoactive substances that, in turn, cause vessels to dilate (Attwell et al, 2010). Maximum vessel dilation in response to a stimulus occurs at the site of greatest neuronal activity and decreases as a function of distance from the centre (Devor et al, 2007); (Blinder et al, 2010). The response is not only graded in amplitude laterally but also retarded in time axially (across cortical depth) so that the dilation of penetrating arterioles in the intermediate layers (IV-V) of the somatosensory cortex precedes that in the top and bottom cortical layers (I - III and VI) (Silva & Koretsky, 2002), (Tian et al, 2010). There is a large body of evidence that identifies the penetrating arterioles as the vessels driving the hemodynamic response (Iadecola & Nedergaard, 2007); (Devor et al, 2007), the arteriolar dilation resulting in increased pressure in the downstream vessels (Boas et al, 2008). The passive dilation and constriction of capillaries in response to increased or decreased upstream pressure has recently been challenged by work showing that pericytes have the potential to actively control capillary diameter (Peppiatt et al, 2006), (Fernández-Klett et al, 2010), (Chen et al, 2011); however capillary, role in neurovascular coupling, if any, is presently unknown (Fernández-Klett et al, 2010). New evidence acquired with improved temporal and spatial resolution suggests that the hemodynamic response is initiated in the parenchyma (Chen et al, 2011), (van Raaij et al, 2011) and not the major vessels. Stimulation induced capillary dilations on the order of 10% have been reported by (Devor et al, 2007), (Stefanovic et al, 2008), with both groups also reporting less frequent stimulation induced capillary constriction, possibly associated with neuronal inhibition, in a spatial pattern that suggests re-distribution of blood supply from dormant to active areas (Kleinfeld et al,
Vascular constriction has also been reported during the post stimulus period (Devor et al, 2007), (Tian et al, 2010), (Chen et al, 2011), where the penetrating arterioles’ diameters briefly drop below pre-stimulation levels after stimulus offset. Interestingly, most groups report no change in venous diameter in response to stimulation (Chen et al, 2011), (Kennerley et al, 2010); in contrast to assumptions made in early models of the hemodynamic response (Buxton et al, 1998), (Mandeville et al, 1999) that predicted significant dilation of compliant veins. This discrepancy is of particular concern because it affects quantitative interpretation of the widely used human neuro-imaging technique BOLD fMRI (blood oxygen level dependent functional magnetic resonance imaging).

Investigation of the mechanism underlying neurovascular coupling on the microvascular network scale is necessary not only as the first step toward the study of neurovascular changes in neurodegeneration, but also as the basis for better modeling of non-invasive macroscopic neuro imaging signal recorded with fMRI which infer neuronal activity from hemodynamics (Berwick et al, 2008). The quantitative interpretation of these macroscopic imaging data requires a model that characterizes the behaviour of neurons and glia as well as the hemodynamic changes in micro and macro-vessels. The current study is novel in that it addresses the spatial and temporal evolution of the response of the 3D microvascular network in vivo under physiological conditions. This work adds to the current literature by estimating the volume changes in the finely sampled microvasculature by imaging the cortex below the superficial layers, without altering intracranial pressure via ventricular puncture and thus compromising baseline hemodynamics, a step widely done in studies to date for logistical purposes.
1.2 Objective

The objective of this work was to measure and map the reactivity of the microvascular network in 3D to stimulation in vivo. Imaging the microvascular network in a physiologically relevant animal model (alpha-chloralose anesthetized Sprague-Dawley rats) was achieved via two photon fluorescence microscopy (2PFM) in the presence of electrical forepaw stimulation. Measurement of the cerebral blood volume (CBV) changes was achieved on a vessel by vessel basis with a semi-automated image analysis pipeline.

1.3 Hypothesis

2PFM in vivo imaging of cerebral cortical microvasculature allows 3D mapping of microvascular response to neuronal stimulation.

1.4 Somatosensory Pathway

Fine sensation from the forelimb is transmitted to the primary somatosensory cortex via three neurons along the dorsal column-medial lemniscus pathway. The primary neuron, extending from forelimb to the brain stem along the dorsal column of the spinal cord, synapses in the fasciculus cuneatus (nucleus) located in the medulla. The secondary neuron decussates and terminates in the ventro-posterolateral (VPL) nucleus of the thalamus. The third neuron extends from the VPL along the internal capsule to layer IV of the somatosensory neocortex located in the post central gyrus. The cortex is a heterogeneous structure where cells are arranged in vertical columns extending through six layers, with layer VI being most inferior. All areas of the cortex have these six layers, but the thickness of each layer is not conserved.
through the cortex (Nolte, 2002). The human cortex is approximately 2 - 4 mm thick (Kandel 2000) whereas the cortex of the adult rat is approximately 1.5 - 2.4 mm thick (Paxinos & Watson, 2007). The incoming sensory signal excites neurons in cortical layer IV first; then the signal spreads toward the surface of the cortex and toward the deeper layers with 3 - 4 ms delay of activation from the onset of activation in layer IV to activation in other layers (Armstrong-James et al, 1992), (Silva & Koretsky, 2002). In layer IV, the columns of neurons function almost entirely separately from one another. Layers I and II receive a diffuse, nonspecific input from lower brain centers. The neurons in layers II and III have axons extending to other related portions of the cerebral cortex, including the contralateral hemisphere through the corpus callosum; while the axons of the neurons in layers V and VI extend to the deeper nuclei of the cerebrum such as the basal ganglia and thalamus (Nolte, 2002).

1.5 Light Interaction with Tissue

Optical imaging of biological tissues is typically limited to the superficial (pial) layer because light is strongly attenuated as a function of depth due to absorption and/or scattering (Helmchen & Denk, 2005). Absorption is the transfer of energy from a photon of light to the tissue, whereas scattering is a deflection of the light due to inhomogeneities in the refractive indices of different tissue compartments (interstitial fluid vs. cytoplasm vs. nucleus vs. intra cellular organelles). Absorption spectra of important tissue components (hemoglobin, water, and lipids) are shown in figure 1. The absorption coefficient is high in the ultraviolet and infrared regions of the spectrum, making absorption the dominant mechanism of attenuation in this frequency range. The absorption coefficient is low in the red and near infrared range: the ‘optical window’ spans 650 nm to 1300 nm; in this frequency range scattering is the dominant
mechanism; nevertheless, light absorption by hemoglobin in major vessels still causes significant signal loss (Kobat et al, 2009). Absorption reduces the intensity of the light traversing the medium, and the energy associated with the absorbed light is usually dissipated as heat in the tissue (Pawley, 2006). The amount of light absorbed is dependent on parameters of the incident light (power, wavelength, exposure time) and the tissue optical properties (absorption coefficient). The amount of heat generated is dependent on thermal properties of the tissue, such as the specific heat capacity. The heating from two-photon water absorption is on the order of 1 mK for typical excitation powers (100 mW average power; 15 kW peak power; 80 MHz repetition; 100 fs pulse duration; 1 fL excitation volume) and has been shown to have negligible biological effects (Kobat et al, 2009).

![Absorption coefficient graph](image)

**Figure 1:** Absorption coefficient (probability of photon absorption in tissue per unit path length) of prominent tissue components within the ‘optical window’. With 810 nm excitation wavelength oxy (O₂Hb) and deoxy (HHb) hemoglobin are the dominant absorbers (reproduced from www.bme.arizona.edu).
The mechanism of scattering is dependent on the size of the scatterer and the light wavelength. Rayleigh scattering, which is inversely proportional to the fourth power of excitation wavelength, is the dominant process when the wavelength is much longer than the diameter of the scatterer (striations in collagen fibrils, macromolecular aggregates, membranes). Mie scattering is the dominant process when the wavelength is on the order of the diameter of the scatterer (mitochondria, lysosomes, vesicles); and is anisotropic as forward scatter increases with particle size (Helmchen & Denk, 2005). Mie scattering is not strongly dependent on wavelength, rather it is proportional to the square of the ratio of refractive indices of the scatterer and the surrounding media (Pawley, 2006). The effective degree of scattering is an average of Rayleigh and Mie scattering and is described by the ‘mean free path’ of a photon, which is the average distance between successive collisions, accounting for anisotropy (Helmchen & Denk, 2005). Maximizing the mean free path of a photon by minimizing interfaces of differing refractive indices (eg. using a water immersion objective) or, in general, choosing a long excitation/emission wavelengths will increase penetration depth as mostly ballistic (unscattered) photons contribute to the two photon microscopy signal. The mean free path of photons through cortical grey matter is approximately 200 μm with 800 nm excitation wavelength (Oheim et al, 2001).

1.6 Two Photon Fluorescence Microscopy (2PFM)

Current imaging modalities for investigating in vivo human brain function (fMRI, positron emission tomography (PET), diffuse optical tomography (DOT)) cannot provide the spatial resolution needed to image individual brain microvessels. Measuring the hemodynamic response of the microvascular network to neuronal stimulation in vivo involves imaging with
sufficient temporal and spatial resolution to capture the hemodynamic changes, induced by neuronal activation, across the three dimensional cortical microvascular network. Two photon fluorescence microscopy (2PFM) allows direct observation of cerebral microvessels in situ at micrometer spatial and millisecond temporal resolution. Over the past two decades, 2PFM has surpassed other optical techniques (eg. confocal microscopy and NIRS) for imaging live brain tissue in animal models due to longer depth of image formation, up to 1 mm (Oheim et al, 2001), which is facilitated by long excitation and emission wavelengths (Pawley, 2006).

The development of lasers over the last several decades, specifically those affording stable pulses (on the order of 100’s of femtoseconds) or mode-locked coherent light, has made 2PFM possible. Within the laser cavity’s gain medium, population inversion is created when the particles are excited to higher and/or metastable energy levels by absorption of energy from a pump source. Some particles experience spontaneous emission and stimulate the particles in the metastable state to emit photons, which are in phase with the incident light. Maximizing the probability of stimulated emission and therefore achieving light amplification requires a resonant cavity, having a highly reflective (99.9 %) mirror at one end and partially reflective (95 %) mirror at the other. The output frequency (mode) of the laser is determined by the distance between the end mirrors, and can be tuned by changing the cavity length. Since many modes can simultaneously oscillate within the cavity, mode-locking to a single frequency is required for pulsed light. Mode-locking results from pulses arising from interference of fixed phase offset modes of the laser’s resonant cavity, and is achieved by introducing additional glass such as an
acousto-optic modulator (AOM) (as described in the next paragraph) into the cavity (Pawley, 2006).

2PFM is a non-linear process that depends on near simultaneous (within $\sim 5 \times 10^{-16}$ sec) (Denk & Svoboda, 1997) absorption of two photons of light by a fluorophore at the focus of the objective. The number of photons absorbed per fluorophore per pulse is given by equation 1 (So et al, 2000):

$$n_a \approx \frac{p_0^2 \delta}{\tau_p f_p^2} \left( \frac{(NA)^2}{2\hbar c \lambda} \right)^2$$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Typical Value</th>
</tr>
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<tbody>
<tr>
<td>$p_0$</td>
<td>average laser intensity 100 mW average</td>
</tr>
<tr>
<td>$\delta$</td>
<td>2-photon cross section $470 \times 10^{-50}$ cm$^4$ sec/photon or 470 Goeppert-Mayer (GM) units</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture 1.05</td>
</tr>
<tr>
<td>$\tau_p$</td>
<td>pulse duration 100 femtoseconds</td>
</tr>
<tr>
<td>$f_p$</td>
<td>repetition rate of laser 80 MHz</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>excitation wavelength 810 nm</td>
</tr>
<tr>
<td>$h$</td>
<td>reduced Plank’s constant $1.05 \times 10^{-34}$ Js</td>
</tr>
<tr>
<td>$c$</td>
<td>speed of light $2.99 \times 10^8$ m/s</td>
</tr>
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A pulsed (typically 100 femtoseconds, 80 MHz) laser is used as the light source to maximize the flux of photons (number of photons moving through unit area per unit time) at the focus, and thus the probability of near simultaneous absorption, while minimizing the energy delivered to the tissue. Mode-locking of the laser is achieved with a standing wave acousto-optic modulator (AOM) which is placed inside the laser cavity close to one end mirror. A time dependent refractive index grating perpendicular to the direction of light propagation is created in the AOM quartz with a piezo-electric transducer causing light to diffract periodically creating pulsatile behaviour. Group velocity dispersion (GVD), which occurs in all optical systems due to wavelength dependent variation in transit time through dispersive media, is particularly problematic with short pulses as it broadens the pulse in time. It is typically compensated for in 2PFM by retarding the transit time of lower frequencies via a series of strategically placed prisms in the light path (Pawley, 2006).

Raster scanning of the sample is conventionally achieved by two mirrors, each driven by a galvanometer which deflects the mirror in response to current flowing through its coil. The numerical aperture (NA) of the objective has a profound effect on image quality as the photon absorption probability, and thus signal amplitude, has a fourth power dependence on it. Maximizing the angle at which light can be emitted and collected (ie. NA), and minimizing refractive index changes by immersing the objective in a refractive index matched medium such as water maximizes SNR in the resulting images (Pawley, 2006).
Two photon molecular excitation consists of the near simultaneous absorption of multiple two photons that combine their energies to cause the transition to the excited state of the chromophore (Helmchen & Denk, 2005). Quantum mechanically, a single photon excites the molecule to a virtual intermediate state, and the molecule is brought to the final excited state by the absorption of the second photon (So et al, 2000). The molecule’s affinity to simultaneously absorb two photons (two photon excitation cross section) is highly dependent on the specific quantum energy selection rules and the excitation wavelength. The fluorophore then emits fluorescence with a wavelength shorter than the exciting laser wavelength within approximately $10^{-9}$ second timescale (Pawley, 2006). The non-scattered emitted photons from the sample traverse through the objective and are guided to the photodetectors via several dichroic mirrors.

Detection, amplification, and digitization of emitted fluorescent signal is most commonly done by photon multiplier tubes (PMT), which electronically amplify the signal. A photon entering the PMT collides with a photocathode resulting in the emission of an electron (photoelectric effect), which accelerates toward and collides with a dynode inducing emission of secondary electrons (secondary emission). The propagation of secondary electrons toward an anode via multiple dynodes and down a voltage gradient results in sufficient amplification of the current for measurement. An example of the light path in a microscope is shown in figure 2.
Figure 2: The light path of an example two photon (pink underlay) microscope includes several dichroic mirrors (RDM690, ADM720, MV/G, MC/Y, MG/R, MDM680), filters (LSM/ R690/ U-MWU2/ U-MNB2/ U-MWG2), galvanometer controlled mirrors, prisms, and external PMTs (BXD1, BXD2).
The spatial resolution in the lateral (xy) and axial (z) planes are given by:

(2) \[ r_{xy} = 0.61\lambda/\text{NA} \]

(3) \[ r_z = 2\eta\lambda/\text{NA}^2 \]

<table>
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<tr>
<th>Parameter</th>
<th>Typical Value</th>
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<td>$\eta$</td>
<td>refractive index</td>
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<td>numerical aperture</td>
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<td>$\lambda$</td>
<td>excitation wavelength</td>
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<td></td>
<td>1.33 for water</td>
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<tr>
<td></td>
<td>1.05</td>
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<td>810 nm</td>
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</tbody>
</table>

The lateral resolution is in practice approximately equal to the lateral resolution of single photon microscopy. The two-photon excitation is confined to a small volume, approximately 0.1 femtoliters ($\mu$m$^3$), resulting in negligible out-of-focus signal (Pawley, 2006).

Despite recent technical advances, 2PFM of the brain is limited because it necessitates focal opening or aggressive thinning of the skull due to its high scattering of light. Successful imaging of cerebral microvasculature thus requires extensive surgical preparation and a flawless craniotomy while maintaining normal physiology. The following chapter outlines the methods employed in this study specifically describing surgical preparation, image acquisition with two photon fluorescence microscopy, and image analysis with a semi-automated pipeline.
2.0 METHODS

2.1 Animal Model

The experimental procedures performed in this study have been reviewed and approved by the Animal Care Committee at Sunnybrook Research Institute, Toronto.

Experiments were conducted on 14 male Sprague-Dawley rats (132 ± 26 g). Animals were prepared for imaging as described in detail in (Lindvere et al, 2010). Briefly, animals were anesthetized with isoflurane (5% induction, 3% maintenance) and a tracheotomy was performed to allow close control of respiratory parameters throughout the experiment. This is especially important because alpha-chloralose is a respiratory and cardiac depressant (Flecknell, 1996). For the remainder of the experiment animals were mechanically ventilated with a mixture of O₂, N₂, and medical air so as to effect a slight O₂ enrichment (FiO₂ between 21 and 24 %). Glycopyrrolate (0.02 mg/kg, IM) and Ringers solution (0.5 - 1 mL/hr, IP) were administered throughout the experiment to decrease secretions and maintain glucose levels and hydration, respectively. The femoral vein and artery were cannulated with catheters made of BPE-T50 tubing (Instech Laboratories Inc., Plymouth Meeting, PA) for administration of anesthesia; and blood pressure monitoring and blood gas analysis, respectively. The tail vein was cannulated for administration of fluorescent agent to allow visualization of the vasculature. Stereotaxic surgery was performed over the forelimb representation of the somatosensory cortex (S1FL) to prepare a small (2.52 to -1.44 mm posterior-anterior to bregma, and 2.75 to 5.5 mm lateral from the midline), closed (1% agarose) cranial window (figure 3). Stimulation electrodes were inserted between the 2nd and 3rd forepaw digits contralateral to the craniotomy and secured with tape, to allow delivery of somatosensory stimuli. Anesthetic was changed from isoflurane to alpha-
chloralose (80 mg/kg induction, 27 mg/kg/hr maintenance) for the remainder of the experiment as neurovascular coupling is relatively well preserved under alpha-chloralose (Ueki et al, 1992). Pancuronium bromide, a muscle relaxant, was administered (2 mg/kg induction, 0.3 mg/kg/hr maintenance) to minimize residual motion. Texas Red (ex: 583/em: 603) dextran (70 kDa) was diluted with PBS (25 mg/ml) and injected through the tail vein catheter (20 mg/kg).

Body temperature, arterial blood pressure, end tidal respiratory pressure, heart rate, and oxygen saturation were monitored and recorded (Biopac Systems Inc., Goleta, CA) throughout the experiment. Arterial blood gases were sampled hourly and adjusted as needed to ensure systemic physiological stability throughout the experiment. Table #1 shows typical values of all physiological parameters measured and describes typical adjustments.

Intracranial pressure (ICP) was measured in a subset of animals (n = 3) to confirm the return of ICP to physiological range following cranial window implantation. After cannulation of the femoral vein and artery as described above, a small (0.84 mm diameter) pressure transducer (Samba 201, Biopac Systems) was introduced into the subarachnoid space of the lumbar vertebrae and sealed such that ICP was measured and recorded for the remainder of the experiment. The ICP decreased by 15 % upon opening of the craniotomy, and returned to within 7 % of pre-craniotomy ICP after closure with 1 % agarose and coverslip.
<table>
<thead>
<tr>
<th>Physiological Parameter</th>
<th>Typical Value</th>
<th>Problem</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracranial pressure</td>
<td>3.7 – 4.4 mmHg</td>
<td>High</td>
<td>Too much trauma to the brain – start again</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>Stop potential leak of cerebrospinal fluid</td>
</tr>
<tr>
<td>Arterial blood pressure*</td>
<td>150/90 mmHg</td>
<td>High</td>
<td>Encourage urination by massaging bladder or increase anesthetic(^\d) dosing if under-anesthetized</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>Increase blood volume by injecting saline or decrease anesthetic(^\d) dosing if over-anesthetized</td>
</tr>
<tr>
<td>Temperature</td>
<td>37 – 38 °C</td>
<td>Low</td>
<td>Use hand warmer, hair dryer, or heat lamp</td>
</tr>
<tr>
<td>Respiratory pressure</td>
<td>10 - 15 cmH(_2)O</td>
<td>High</td>
<td>Check for plugging of tracheal tube (obstruction) or decrease flow rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>Increase flow rate</td>
</tr>
<tr>
<td>pH (measured by arterial blood gas analysis)</td>
<td>7.35 - 7.45</td>
<td>Low</td>
<td>Administer sodium bicarbonate (IV) according to base excess (ABEc): if ABe &lt; -5 mmol/L then administer (</td>
</tr>
<tr>
<td>Heart rate</td>
<td>320 – 450 bpm</td>
<td>High</td>
<td>Increase anesthetic(^\d) dosing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>Decrease anesthetic(^\d) dosing</td>
</tr>
<tr>
<td>Oxygen saturation</td>
<td>96 – 99 %</td>
<td>Low</td>
<td>Increase FiO(_2) or decrease anesthetic(^\d) dose</td>
</tr>
<tr>
<td>pCO(_2)</td>
<td>30 - 36 mmHg</td>
<td>High</td>
<td>Increase breath rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>Decrease breath rate</td>
</tr>
</tbody>
</table>

Table 1: Physiological parameters monitored during surgery and imaging to ensure physiologically stable state is maintained. Typical values are found in (Nagra et al, 2009), (Waynforth & Flecknell, 1992), (Flecknell, 1996), and (Hutchinson et al, 2006).
\(^\d\)Assuming halogenated anesthetic (eg. isoflurane).
\(^*\)Absolute range of arterial blood pressure depends on the level of anesthesia.
Figure 3: A: Closed cranial window preparation with a dental cement well to hold water for water immersion objective. B: Open cranial window prior to closing with coverslip.

2.2 Two Photon Fluorescence Microscopy (2PFM)

Two photon fluorescence microscopy was performed using a twin system FV 1000 Multi Photon Excitation Microscope (Olympus Corp., Tokyo, Japan) equipped with group velocity dispersion compensation unit, acousto-optic modulator (AOM), and a water immersion objective (25X, 1.05NA, 2 mm WD). Pulsed near infrared light ($\lambda_{ex} = 810$ nm) was generated via Mai Tai MP Ti:Sapphire pulsed, tunable (690 - 1040 nm) laser (Spectra Physics, Santa Clara, CA). Emitted fluorescence $\lambda_{em}$, in the 570 - 625 nm range, was collected using a dichroic filter (FV10MP - MG/R) and an external PMT detector (R6357, Hamamatsu). The average intensity of the input light was increased as a function of depth, every 50 $\mu$m keeping the total number of pixels saturating constant and interpolating linearly in between, to counteract exponential signal attenuation due to light scattering in tissue (only linear interpolation was available on this platform). The
average energy at the surface of the brain as measured by power meter sensor (Newport, Irvine, CA) ranged from ~ 20 mW when imaging superficial vessels to ~ 130 mW when imaging vasculature ~ 600 μm below the cortical surface (figure 4).

Figure 4: Imaging of fluorescently labeled plasma is achieved after implantation of a closed cranial window. The well serves as a basin for water to immerse the high NA (1.05) 25 X objective for two-photon fluorescence microscope imaging (2PFM).

2.3 Point Spread Function (PSF)

The point spread function (PSF) describes the response of an imaging system to a point object and is useful for image deconvolution, resulting in a sharper image. PSF was measured for the
2PFM used in the current work with a sub-resolution (0.17 µm diameter) fluorescent bead fixed to a glass slide and was found to be ellipsoidal with the full width half maximum (FWHM) of 0.33 µm in x, 0.40 µm in y, and 1.36 µm in the z direction (figure 5).

![Intensity profiles](image)

**Figure 5:** The intensity profiles (lateral x; and axial z) of a sub-resolution (0.17 µm diameter) fluorescent bead acquired with 25 X magnification and 1.05 NA. The full width at half max (FWHM) is estimated (by Gaussian fit - red) to be approximately 0.33 µm in x, and 1.36 µm in z).

### 2.4 Image Acquisition

Two types of acquisition were performed:

i. Fine spatial resolution stacks (FOV: 508 µm², lateral resolution: 0.994 µm/pix, dwell time: 8 µs/pix) were acquired for visualization of vascular network architecture. A dynamically modified weighted-average filter (Kalman) was used to increase SNR (Anderson and Moore,
1979). This acquisition reached a depth of 600 µm below the pial surface with an axial resolution of 3 µm/pix requiring approximately 20 minutes to complete.

ii. Functional data were acquired at coarser spatial resolution (FOV: 508 µm², lateral resolution: 1.59 µm/pix, dwell time: 4 µs/pix, with no averaging) to improve temporal resolution. Three stimulation paradigms were presented:

Paradigm 1: The depth of imaging was maximized (0 - 600 µm). The acquisition paradigm, analogous to leaved fMRI protocols (Silva & Koretsky, 2002), was automated using Olympus’ Time Controller environment such that 15 time frames of a 2D slice at each cortical depth were acquired with temporal resolution of 0.786 sec/frame. The stimulation, triggered at the beginning of each frame, was OFF for frames 1 and 2, ON for frames 3 to 5, and OFF for frames 6 to 15. Each ‘ON’ block consisted of 7 pulses (2 mA in amplitude, 0.3 ms in duration) of electrical forepaw stimuli delivered at 3 Hz. This paradigm was chosen to maximize the amplitude of the cerebral blood volume (CBV) response, which has been shown to saturate after 6 pulses (Hirano et al, 2011), while minimizing acquisition time. After the most superficial slice was acquired with this paradigm, the imaging depth was increased by 6 µm and the same functional paradigm was repeated. This was first done for z levels corresponding to even depths (0, 6, 12 µm, ... 600 µm) and then repeated for z levels corresponding to odd depths (3, 9, 15 µm, ... 597 µm). These 200 15-frame time series acquired at increasing depths thus provide a coarse spatial resolution stack (508 µm × 508 µm × 600 µm with lateral resolution of 1.59 µm/pixel and axial resolution of 3 µm/pixel) effectively sampled every 0.786 seconds, under the assumption of temporal invariance of the hemodynamic response to stimulation.
Paradigm 2: The depth of imaging was confined to 100 μm - 400 μm to probe the epicentre of microvascular response in cortical layers II/III. The acquisition was analogous to Paradigm 1; however, this stimulus presentation was triggered by the microscope so that it was ON for frames 3 to 5, and 24 to 26, and OFF for frames 1 and 2, 6 to 23 and 27 to 42. Each ‘ON’ block again consisted of 7 pulses (2 mA in amplitude, 0.3 ms in duration) of electrical forepaw stimuli delivered at 3 Hz. These 100 42-frame time series acquired at increasing depths thus provide a coarse spatial resolution stack (508 μm × 508 μm × 300 μm with lateral resolution of 1.59 μm/pixel and axial resolution of 3 μm/pixel) effectively sampled every 0.786 seconds, again under the assumption of temporal invariance of the hemodynamic response to stimulation (figure 6).

Paradigm 3: To probe the impulse response, the depth of imaging was confined to 100 μm - 400 μm and acquisition was analogous to Paradigm 1; however, this stimulus presentation was triggered such that a single pulse (2 mA in amplitude, 0.3 ms in duration) was delivered at the beginning of frames 3, 11, 19, 27, and 35. These 100 40-frame time series acquired at increasing depths thus provide a coarse spatial resolution stack (508 μm × 508 μm × 300 μm with lateral resolution of 1.59 μm/pixel and axial resolution of 3 μm/pixel) effectively sampled every 0.786 seconds, again under the assumption of temporal invariance of the hemodynamic response to stimulation.
Figure 6: 2D planar data is acquired over time in sequential depth (100 - 400 µm), then reconstructed to volumes with spatial resolution of 1.6 x 1.6 x 3 µm and effective temporal resolution of 0.786 sec/volume. Stimulus in paradigm 2 is applied during frames 3, 4, 5 and 24, 25, 26 (paradigm repetition not shown).

2.5 Analysis

Radii changes are expected from stimulus induced hyperemia associated hemodynamic adjustments, autoregulation, and vasomotion (spontaneous oscillations (< 0.1 Hz) of arterial and arteriolar radii (Kleinfeld et al, 1998), but not capillary radii (Fernández-Klett et al, 2010). Displacement may be induced by cardiac and/or respiratory cycles, and brain pulsations. The data were structured as a set of vertices characterized (x, y, z, r) at each time instance. The mean distance between neighbouring vertices was 0.78 ± 0.13 µm, meaning that several vertices made up a vessel, defined as the segment between two branch points. To estimate vessel volume across the vascular network at each time frame, while establishing vertex correspondence across time, the data analysis was done as follows (figure 7):

i. The anatomical data were processed in the following way:

a. The characteristic attenuation length (CAL) was calculated as described in (Kobat et al, 2009). Briefly, the logarithm of the average value of the brightest 10 % of the pixels from each frame were plotted as a function of depth. The characteristic attenuation length is defined as the depth at which there is an $e^{-2}$ fold decay in the normalized...
detected fluorescence signal. The CAL measurement was used to assess the quality of the image.

b. The anatomical data were segmented using semi-automated analysis via commercially available software (Imaris, Bitplane, Zurich). Prior to segmentation, the data were subjected to edge-preserving 3D anisotropic diffusion filtering (Perona & Malik, 1990). The intravascular space was identified based on a range of user supplied signal intensity thresholds (defining the background and foreground signal intensity ranges). The resulting volumes were next skeletonized, with the network sampled every 1 µm, and the data structure containing vertex wise coordinates (x, y, and z), their corresponding radius estimates (r), and their connections. This filament object was then converted to a graph, and the local tangents to the vessel evaluated at each vertex following spline fitting to the vertex locations (figure 8).

c. The segmentation of the vasculature was used to estimate the cerebral blood volume (CBV). This measure estimated the CBV as the luminal volume as fluorescent dextran distributes within plasma. The CBV was an underestimation because the imaging FOV was chosen to not include major pial vessels which have major contributions to the CBV, but severely attenuate the signal and render deep imaging impossible.

ii. The coarse resolution functional data were processed in the following way:

a. restitch the raw data such that a 3D volume is created for each time point

b. filter each slice with a 2D Gaussian kernel (σ is 1.2 in x, 1.2 in y)

c. register (automated) each 2D time series to its temporal mean (2dImReg, AFNI suite)

d. register (manually) even slices to odd slices via landmark identification
e. normalize signal intensities (using ratio of medians to decrease sensitivity to outliers) through the cortical depth (iNormalize, AFNI suite)

f. register (finely and manually) the temporal mean of functional volumes to anatomical data using full affine transformation and a set of manually identified landmarks

g. resample functional data to isotropic coordinates for radius estimation algorithm

h. mask the anatomical space with the functional field of view

i. compute absolute foreground/background intensity range in each functional volume for the purposes of the proceeding analysis

j. estimate vascular radius and centre line position on the temporal mean of functional volumes using in-house algorithm. Filament object from Imaris segmentation of the anatomical data (as described in section 2.5 i.b.) was used as an initial guess for the location and radii of vertices at each time point figure 9 (left). A model based segmentation algorithm inspired by (Fridman et al, 2004) was used to estimate vertex-wise locations and vascular radii on the temporal mean of the functional data (c). Briefly, the data were smoothed with a kernel whose sigma (σ) is set to 40% of the vertex’s current radius estimate. Eight ‘spokes’ of length equal to this radius are defined in the plane normal to the local tangent to the vessel, radiating outward from each vertex. At the tip of each spoke, the gradient of signal intensity is computed in the direction of the spoke. The objective function is defined as the maximum sum of six, out of eight (to facilitate tracking at branch points), finite differences between intra and extra luminal points (whisker operator). To ensure computational efficiency, regularization is done on both vertex displacement and radius change. The vascular cross-section is assumed to be circular while the PSF is assumed to be anisotropic as
determined a-priori, see section 2.3. This optimization allows estimation of the vertex-wise locations and radii while establishing vertex correspondence across time. In the final step, the tangents are re-evaluated in light of new vertex locations. This results in a graph file containing vertex wise coordinates \((x, y, z)\), radius estimates \((r)\), and tangents \((\theta)\) for the temporal mean of the functional 3D vascular network (figure 9 and 10).

k. re-estimate the vascular radius and centre line position on the functional volume at each time point using the same algorithm as described in step j. This results in multiple graph files containing vertex wise coordinates and estimates \((x, y, z, r, \text{ and } \theta)\) for each time point. To ensure graph structure consistency in time, the radii data were applied across time to a unique vascular network morphology (second time frame), as identified by the set of coordinates \((x, y, z, \text{ and } \theta)\).

l. perform generalized linear model (GLM) analysis incorporating a modeled hemodynamic response function (HRF) and a first order polynomial to account for baseline variations (AFNI: 3dDeconvolve; (Cox, 1996)). The HRF was based on the blood volume response measured via contrast enhanced functional MRI in the same animal model using the same functional paradigm (Hirano et al, 2011) and was defined using AFNI: WAVER (Cox, 1996). Three separate HRFs were used to probe for quick, mid-latency, and slow responses to stimulus, having onset times of: 0 sec, 0 sec, 0.776 sec, times-to-peak of: 2.4 sec, 3.9 sec, 4.7 sec, and FWHMs of: 3.0 sec, 3.3 sec, 3.9 sec respectively.

m. the radii vertex wise data are interpolated in space to maximize connectivity of resulting data set and tangents are subsequently re-estimated.
n. compute, via AFNI: 3dClustSim, the cluster sizes for given cluster-wise alpha
(probability of a noise-only smooth random field), here set at 0.05, after masking and
then thresholding at the given per-vertex p value, here set at 0.05, and given the
specified amount of smoothness in the image time series noise, as determined by the
ratios of variance of first differences to de-trended data variance, with smoothness
level estimated via the mean geometric FWHM along each axis (3dFWHMx: AFNI
suite). For each of the three HRF models, we produced cluster maps corresponding to
third-nearest neighbour clustering (faces or edges or corners touching).

o. mask the effect sizes (radii changes) from GLM by the clustering-based masks to show
radius changes of activated clusters.
Figure 7: Data processing pipeline. Functional volumes are subjected to motion correction, Gaussian filtering, and intensity normalization across cortical depths prior to location, tangent, and radius estimating algorithm. General linear model analysis is done with AFNI (Cox, 1996).
Figure 8: A: 3D rendering of 2PFM image of cortical microvasculature in forelimb representation of the primary somatosensory cortex B: Segmentation of 2PFM image done with commercially available software, Imaris (Bitplane).

Figure 9: View of the plane orthogonal to the longitudinal vessel axis. A: initial guess based on Imaris segmentation of the anatomical acquisition (figure 8b). B: final estimate using in-house multi-scale algorithm allowing small adjustments to centre line location and radius value.
Figure 10: Radius and position estimation from one time point using in-house algorithm segmentation (red) overlaid on temporal mean of functional data. Left: x-y slice (parallel to cortical surface) approximately 150 µm below superficial pial layer. Right: x-z (top) and y-z (bottom) slice. Profile of cortical vasculature between 100 to 400 µm includes many penetrating vessels.
3.0 RESULTS

3.1 Anatomical Data

3.1.1 Characteristic Attenuation Length (CAL)

The anatomical images were used to calculate the characteristic attenuation length (CAL), which is defined as the depth at which there is an $e^{-2}$ fold decay in the normalized fluorescent signal, in the brightest 10% of pixels, as described in (Kobat et al, 2009). The CAL was estimated to be $372 \pm 87 \mu m$ $(n = 9)$ for an excitation wavelength of 810 nm (figure 11). Data sets with CAL less than 200 $\mu m$ were considered to be of poor quality, and therefore not included in further analysis.

![Characteristic Attenuation Length](image)

Figure 11: The characteristic attenuation length (CAL) curve of an example data set. CAL is defined as the depth at which there is an $e^{-2}$ fold decay in normalized fluorescent signal of the brightest 10% of pixels as described in (Kobat et al, 2009). CAL is estimated at 375 $\mu m$ for this data set.
3.1.2 Total Cerebral Blood Volume (CBV)

The semi-automated segmentation of the anatomical images using Imaris (as describe in section 2. 5 i.b. and shown in figure 8B) was used to estimate the total cerebral blood volume (CBV). The total luminal CBV was estimated to be 3.4 % ± 1.6 % (n = 9). This estimate included vessels less than 35 μm in radius, ie. avoided major pial vessels.

3.2 Functional Data

A brief note on the structure and processing of functional data: three paradigms were used as described in section 2.4. The functional data were comprised of a set of vertices characterized (x, y, z, r) at each time instance. These data were subject to general linearized modeling of radius as a function of time using three hemodynamic response (HRF) models. Three HRFs were specified so as to probe for quick, mid-latency, and slow responding vascular vertices. Vertices were deemed ‘active’ if they passed the threshold of p < 0.05 and cluster wise α < 0.05. Activated vertices required a minimum cluster size of 25, based on a random-noise simulation which suggested that given the number of vertices in the data, random noise thresholded at a vertex-wise p-threshold of 0.05 would yield a false-positive cluster of 25 vertices 5.0 % of the time (AFNI: 3dClustSim). The mean distance between neighbouring vertices was 0.78 ± 0.13 μm, meaning that several vertices made up a vessel, defined as the segment between two branch points. We have done vertex-wise analysis to describe the microvascular network behaviour since the vessels response is heterogeneous along the longitudinal axis of a vessel. Thereafter, for comparison, we also analyzed the response of the vessels. The effect of resting vascular parameters (resting radius, vertex cortical depth, and HRF type) on absolute and
relative radius change was probed using a linear mixed-effects model (Baayen et al, 2008). (A comprehensive list of results can be found in the appendix)

**3.2.1 Paradigm 1**

The stimulation induced hemodynamic response of the vasculature (distribution of vessel size is shown in figure 12) was scrutinized in the data of functional paradigm 1 that spanned from the cortical surface (but still avoided major pial vessels) to 600 μm below in two subjects. The normalized dilation and constriction of vertex and vessel radii was found to be inversely proportional to the resting radius (p < 0.05) (regardless of HRF type), suggesting that the microvasculature underwent the largest relative changes in CBV. Amplitude of dilation and constriction of vertices depended significantly on depth such that larger relative dilations occurred deeper in the cortex (p < 0.05). The net change in volume as a result of dilating or constricting vessels was calculated; although the large vessels had small relative changes in vertex and vessel radius, their absolute contributions to the overall network change in CBV were proportional to their rest volume (p < 0.05). The normalized dilation and constriction of vertex radii was positively correlated with cortical depth (p < 0.05) suggesting that the largest normalized (with respect to rest radius) CBV changes were occurring between 100 to 400 μm deep in the cortex (figure 12) of all vertices with all HRFs. This motivated us to probed the hemodynamic response of the microvasculature over this depth of interest ‘sub - volume’.
Figure 12: Distribution of vessels from 0 to 600 µm data set (n = 2) (left). Major pial vessels were avoided. Imaging the full available depth (0 to 600 µm), majority of dilations and constrictions were seen between 100 to 400 µm (right), motivating further scrutiny of this depth of interest.

3.2.2 Paradigm 2

Within the sub-volume (100 to 400 µm) the range of vertex and vessel radii was decreased, such that approximately 91 % of vessels in the imaging FOV were microvessels (r < 5.0 µm), 7 % had a radius between 5 to 10 µm, and the remaining 2 % had a radius between 10.5 to 30 µm as seen in figure 13. Again, the normalized change in vertex and vessel radii was inversely proportional to the resting radius (p < 0.05). This was true of both dilation and constriction.
**Figure 13: Distribution of vessels in sub-volume (cortical depth from 100 to 400 µm) (n = 7).**

The vertex depth was found to depend on the HRF type (p < 0.05): mid-latency and slowly dilating vertices were located more superficially than quickly dilating vertices. Furthermore, mid-latency constricting vertices were closer to the cortical surface than quickly constricting vertices. Moreover, the relative dilation of vertices and vessels was found to depend on their depth (p < 0.05) with larger relative changes occurring in shallower vessels. Vertex constrictions were found to be dependent on depth (p < 0.05), again with larger relative constrictions occurring in shallower vertices.

### 3.2.2.1 Temporal Response of Paradigm 2

The average time traces of activated vertices from paradigm 2 are shown in figure 14. The time traces of each ‘active’ vertex were first averaged within a subject and then across subjects (n = 7) for dilating and constricting vertices, within each of the three defined modeled HRFs (quick, mid-latency, and slow). The two repetitions of the average time traces were then averaged and
fit using a Gamma variate model (figure 14) to estimate the amplitude, onset time (OT), time to peak (TTP), and full width at half maximum (FWHM) for each HRF (Table 2). The onset time was measured from the Gamma variate fit as the first of two consecutive points that were greater than 10% of the peak amplitude after the beginning of stimulation. The OT of dilating and constricting vertices increased from quick to mid-latency to slowly responding vertices. Dilation lagged constriction in the quick and mid-latency responses, but lead constriction in the slow response. The amplitude of dilation of slow responding vertices was larger than the amplitude of mid-latency and quick responding vertices; in contrast, the amplitude of constriction of slow responding vertices was smaller than the amplitude of mid-latency and quick responding vertices. The TTP, also quoted relative to the beginning of stimulation, and FWHM of both dilating and constricting vertices increased from quick to mid-latency to slow responses.

<table>
<thead>
<tr>
<th>Response</th>
<th>OT (sec)</th>
<th>Amplitude (Δr in %)</th>
<th>TTP (sec)</th>
<th>FWHM (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quick</td>
<td>Dilation</td>
<td>0.00</td>
<td>1.23 ± 0.04</td>
<td>2.78 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Constriction</td>
<td>0.00</td>
<td>-1.30 ± 0.07</td>
<td>2.77 ± 0.03</td>
</tr>
<tr>
<td>Mid-Latency</td>
<td>Dilation</td>
<td>0.14</td>
<td>1.37 ± 0.06</td>
<td>3.98 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Constriction</td>
<td>0.10</td>
<td>-1.19 ± 0.03</td>
<td>3.99 ± 0.02</td>
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<tr>
<td>Slow</td>
<td>Dilation</td>
<td>0.84</td>
<td>2.00 ± 0.06</td>
<td>5.12 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Constriction</td>
<td>1.12</td>
<td>-1.09 ± 0.03</td>
<td>5.52 ± 0.03</td>
</tr>
</tbody>
</table>

Table 2: Temporal parameters of the average hemodynamic response to stimulus paradigm 2 of quick, mid-latency, and slow responding vertices as estimated via Gamma variate fit. Values are reported as mean ± 95% confidence interval.

The time course of the mid-latency dilating vertices suggests that there may be a post-stimulus undershoot; however, it was not explored presently as it is not the subject of the current body of work.
Volume of a vessels was calculated assuming a straight cylinder, therefore the cerebral blood volume (CBV) is proportional to the length of the vessels and square of the radius. Relative changes in volume (figure 16) are, not surprisingly, inversely proportional to vessel resting radius ($p < 0.05$); however, absolute changes are proportional to resting radius ($p < 0.05$), meaning that small vessels have large relative gains and losses in total CBV, but large vessels contribute large absolute gains and losses in CBV. The net change in absolute CBV was positive for quick and mid-latency responding vessels, but negative for slow latency responders.
Figure 14: A, B, C: Average vertex-wise timecourse of paradigm 2 across subjects for dilations (red), constrictions (blue) and modeled HRF (black). Stimulation presentation is shown in yellow. The mean is shown in bold with the inter-subject standard deviation shaded for quick, mid-latency, and slow responding vertices. D, E, F: Gamma variate fit to average timecourses for quick, mid-latency, and slow responding vertices. Active vertices were first averaged within each subject, then averaged across subjects (n = 7), and finally averaged across the two presentations of the stimulus.
3.2.3 Paradigm 3

Paradigm 3 comprised five repetitions of a single stimulation pulse, which were modeled using the quick and mid-lagety HRF only (the inter stimulus interval was too short to employ the slow latency HRF). In agreement with paradigms 1 and 2, the normalized change in vertex and vessel radii was inversely proportional to the resting radius ($p < 0.05$). This was true of both dilations and constrictions. The relative change in vertex radius was significantly larger (for both dilations and constrictions) in quickly responding vertices when compared to mid-latency response vertices ($p < 0.05$).

3.2.3.1 Temporal Response of Paradigm 3

The average time traces of activated vertices from paradigm 3 are shown in figure 15. Analogous to the processing steps used to analyze the temporal response of Paradigm 2, the time traces of each ‘active’ vertex were first averaged within a subject and then across subjects ($n = 7$) for dilating and constricting vertices, within two defined modeled HRFs (quick and mid-latency). Two subjects exhibited poor SNR in response to the quick HRF, likely due to inconsistent physiology, and were not included in the analysis. The five repetitions of the stimulus presentation were then averaged and fitted using a Gamma variate model (figure 15) to estimate the amplitude, onset time (OT), time to peak (TTP), and full width at half maximum (FWHM) for each HRF (Table 3). The onset time (OT) of quick and mid-latency responding vertices was estimated to be instantaneous. The amplitude of dilating vertices increased from quick to mid-latency responders, whereas the amplitude of constricting vertices remained relatively constant. The time-to-peak (TTP) increased from quick to mid-latency responding
vertices, and furthermore, TTP of dilating vertices lead TTP of constricting vertices. Similarly, the full width at half maximum (FWHM) increased from quick to mid-latency responding vertices. The FWHM of quickly dilating vertices was greater than that of quickly constricting vertices: however, FWHM of mid-latency dilating vertices was less than that of mid-latency constricting vertices.

<table>
<thead>
<tr>
<th></th>
<th>OT (sec)</th>
<th>Amplitude (Δr in %)</th>
<th>TTP (sec)</th>
<th>FWHM (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Quick</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilation</td>
<td>0.000</td>
<td>0.969 ± 0.030</td>
<td>0.098 ± 0.013</td>
<td>2.242 ± 0.057</td>
</tr>
<tr>
<td>Constriction</td>
<td>0.000</td>
<td>-0.996 ± 0.021</td>
<td>0.103 ± 0.012</td>
<td>1.946 ± 0.043</td>
</tr>
<tr>
<td><strong>Mid-Latency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilation</td>
<td>0.000</td>
<td>1.207 ± 0.022</td>
<td>0.963 ± 0.007</td>
<td>2.771 ± 0.031</td>
</tr>
<tr>
<td>Constriction</td>
<td>0.000</td>
<td>-1.074 ± 0.020</td>
<td>1.353 ± 0.009</td>
<td>2.952 ± 0.036</td>
</tr>
</tbody>
</table>

Table 3: Temporal parameters of the average hemodynamic response to stimulus paradigm 3 of quick and mid-latency responding vertices as estimated via Gamma variate fit. Values are reported as mean ± 95% confidence interval.
Figure 15: A, B: Average vertex-wise time course of paradigm 3 across subjects for dilations (red), constrictions (blue) and modeled HRF (black). Stimulus presentation is shown in yellow. The mean is shown in bold with the inter-subject standard deviation shaded. C, D: Gamma variate fit to average time courses for quick and mid-latency responding vertices. Active vertices were first averaged within each subject, then averaged across subjects (n = 5 for quick HRF; n = 7 for mid-latency HRF), and finally averaged across the five presentations of the stimulus.

The outcome from the volume estimates are similar to those of paradigm 2: relative volume dilations were inversely proportional to vessel resting radius (p < 0.05). The absolute dilation
was proportional to the vessel resting radius (p < 0.05). The net effect of the stimulation on the CBV was positive.

Figure 16: Map of estimated stimulation-induced change in radius ($\Delta r$) for paradigm 2, dilations (red) and constrictions (blue) of a single subject for quick, mid-latency, and slow latency responses (left). Regions of statistically significant correlation after clustering (right) are shown for each HRF response. Scale bar in green (bottom right) is 100 µm.
3.3 Map of Microvascular Response to Stimulation

Figure 16 shows the microvascular response to stimulation Paradigm 2 for the three defined HRFs (quick, mid-latency, and slow). Un-masked change in radii are shown on the right, while the t - value for statistically significant clusters are shown on the left. White arrows high-light dilation of penetrating vessels corresponding to the quick HRF, and constriction of the same vessels corresponding to the slow HRF.

3.4 Conclusion

We have demonstrated that stimulation induced hemodynamic response of the microvasculature can be mapped using 2PFM imaging. The response of the cerebral microvasculature was, as expected, heterogeneous.
4.0 DISCUSSION AND FUTURE WORK

4.1 Discussion

We have demonstrated how the stimulus induced hemodynamic response of the 3D microvascular network can be mapped using 2 photon fluorescence microscopy. 2PFM’s unsurpassed in vivo spatial resolution (approximately 1 μm x 1 μm x 3 μm in this study) in combination with the fMRI inspired experimental protocol (both with respect to image acquisition and stimulus presentation) provides new insight into cerebrovascular topology and behaviour. The preprocessing steps and generalized linear modeling, expand the scope of 2PFM studies to date, notwithstanding the need for optimization of some of the processing steps (eg. clustering) for the vascular tree topology.

The characteristic attenuation length (CAL) was estimated to be 372 ± 87 μm for excitation wavelength of 810 nm. This is higher than the 131 μm and 285 μm reported by (Kobat et al, 2009) for 775 nm and 1280 nm excitation wavelength, respectively. The increased CAL in this work may be due to the continuous increase in laser intensity as a function of depth compared to just one power adjustment in the aforementioned study. The current study also minimized the attenuation of the signal by avoiding imaging the vicinity of large pial vessels which lead to pronounced absorption of light by hemoglobin and scatter of light from refractive index changes at vessel walls. In the current study the laser power was measured at the surface of the sample to be about 130 mW when imaging 600 μm deep into the cortex with the 810 nm excitation wavelength. In the former study, 93 mW was measured at the surface when imaging 1000 μm deep in the cortex using 1280 nm excitation wavelength.
The estimated luminal CBV of 3.4 % ± 1.6 % in this work is in agreement with the 4.6 % ± 0.4 % previously reported as the CBV of mice measured from ex-vivo micro-CT imaging (Chugh et al, 2009).

The estimation of the change in radii of vertices and vessels was extended to volume assuming constant vessel length. Although focal dilations of up to 15 % were observed in vertices, average dilation amplitudes for quick, mid-latency, and slow responding vertices in response to stimulation Paradigm 2 were estimated via Gamma variate fitting to be 1.2 %, 1.4 %, and 2.0 % respectively. The volume increases associated with these dilations are approximately 2.5 %, 2.8 %, and 4.0 % respectively, much lower than approximately 11 % as measured with 2PFM by (Stefanovic et al, 2008) in the somatosensory cortex for alpha-chloralose anesthetized rats receiving 60 seconds of forepaw stimulation, and lower than 10.4 ± 3.3 % as measured using contrast enhanced fMRI in cortical layers I - III in alpha-chloralose anesthetized rats receiving 2 seconds of forepaw stimulation (Hirano et al, 2011). The measurement by Hirano et al. may be dominated by large dilations of pial vessels, which are avoided in the current study. The fMRI estimation of volume increase in response to a stimulus is an average within a voxel that includes both dilating and constricting vertices and vessels. The average constrictions for quick, mid-latency, and slow responding vertices as estimated in this study by Gamma variate fitting were 1.3 %, 1.2 %, and 1.1 % respectively, although focal instances of constrictions up to 15 % were observed. The decreases in relative volume associated with constriction were 2.6 %, 2.4 %, and 2.2 % respectively. Interestingly, the amplitude of dilation in response to stimulation Paradigm 3 (impulse response) of up to 2.4 % is consistent with 2.9 ± 1.2 % as reported by
Hirano et al. in 2011 in response to a single stimulation pulse in alpha-chloralose anesthetized rats as measured by fMRI.

A systematic variation in this study is the precise placement of the imaging FOV, which was chosen on a subject-by-subject basis by considering the transparency of the imaging region, and may have varied (centre-to-centre) by up to 1.5 mm. Since the amplitude of the vascular response decreases as a function of distance from the epicentre of neuronal response (Devor et al, 2007), the low average radius changes may have resulted from variable location of the relatively small (508 μm x 508 μm) FOV and, furthermore, the inability to average laterally within each cortical layer as is done in fMRI. Finally, the absolute results of 2PFM are severely affected by the artery to vein ratio within the FOV, as the ratio in the primary somatosensory cortex is typically 2 penetrating arteries to 1 penetrating vein.

SIZE DEPENDENCE
Percent and absolute dilations and constrictions are larger in small vessels. Large vessels can make large contributions to total CBV with small percent and absolute changes in radius in response to stimulation.

QUICK VS SLOW LATENCY RESPONDERS
Our findings suggest an upward propagation of dilation and constriction as quickly responding vertices lie deeper than mid-latency and slowly responding vertices. This is in accordance with other groups’ observations (Hirano et al, 2011), (Devor et al, 2007), and propagation of neuronal activation which spreads outwardly from cortical layer IV (Nolte, 2002).
Slowly responding vessels have smaller relative and absolute dilations than quickly responding vessels suggesting that the putative arteriolar side of the circulation makes a larger contribution to CBV change than the venous side. Similarly, slowly responding vessels have smaller constrictions than quickly responding vessels. This could be the result of a post stimulus undershoot being restricted to the arterioles; those vessels that dilated quickly, subsequently constricted.

RESPONSE KINETICS (VERTEX WISE)

Paradigm 2

The difference between the time-to-peak (TTP) of dilating and constricting vertices was largest in the slow responding vessels implying a more sluggish response in the latter phase of vascular constriction. Our estimates of the time to peak of dilating vertices range from 2.8 to 5.1 seconds and are consistent with $2.8 \pm 0.3$ seconds measured with functional micro-ultrasound (fMUS) (van Raaij et al, 2011) and $2.8 \pm 0.3$ seconds measured with contrast enhanced fMRI (Hirano et al, 2011); both groups imaged the somatosensory cortex of alpha-chloralose anesthetized rats stimulated with a functional paradigm analogous to ours; however, they report only on increases in CBV as a result of dilation and do not report on loss of CBV as a result of constriction. Secondly, the retarding of the onset times of constricting vertices with respect to dilating vertices also implies a sluggish response in the latter phase of vascular constriction. The onset times for dilating vertices ranging from 0 to 0.85 seconds agree with $0.9 \pm 0.4$ seconds measured by fMUS for 2 second forepaw stimulation (van Raaij et al, 2011) and $0.3 \pm 0.2$ seconds as measured by fMRI for 2 second forepaw stimulation (Hirano et al, 2011);
again in alpha-chloralose anesthetized rats. The onset time for constriction range from 0 to 1.12 seconds. Thirdly, the larger increase in FWHM of slowly constricting vertices when compared to slowly dilating vertices suggests a sluggish response in the latter phase of vascular constriction. The FWHM of dilating vertices as measured in this work range from 3.4 to 3.7 seconds, and agree with 3.4 ± 1.6 seconds as measured with fMRI by (Hirano et al, 2011).

Paradigm 3: Impulse Response

The onset times (OT) as estimated via Gamma variate fit as 0 seconds for both quick and mid-latency responding vertices is shorter than 0.3 ± 0.2 seconds as measured by fMRI for single pulse forepaw stimulation (Hirano et al, 2011) in alpha-chloralose anesthetized rats. The radial amplitude change of quick and mid-latency dilating vertices measured as 0.97 ± 0.03 % and 1.21 ± 0.02 %, respectively correspond to a volume increase of approximately 2.0 % and 2.4 % respectively, which is consistent with 2.9 ± 1.2 % as reported by Hirano et al. in 2011. In the aforementioned study Hirano et al. reported TTP of 1.0 ± 0.2 seconds in response to a single stimulation pulse, which is higher than the TTP of quickly dilating vertices as measured in the current work, but in agreement with 0.96 ± 0.01 seconds (mean ± 95 % confidence interval) of mid-latency dilating vertices as measured in the current work. The TTP of constricting vertices lagged the TTP of dilating vertices, with the most pronounced difference in the mid-latency responders, which is consistent with sluggish behaviour of constricting vertices from Paradigm 2 as discussed above. The FWHM for quick and mid-latency dilating vertices ranging from 2.24 ± 0.07 to 2.77 ± 0.03 seconds as estimated in the current study are longer than 1.0 ± 0.1 seconds as measured by Hirano et al. 2011 in response to a single stimulation pulse. The FWHM of quickly dilating vertices was greater than that of quickly constricting vertices: however, FWHM
of mid-latency dilating vertices was less than that of mid-latency constricting vertices suggesting a sluggish response of mid-latency constricting vertices, again in agreement with the response from Paradigm 2 as discussed above.

CORTICAL DEPTH EFFECTS (VOLUME)

Our findings suggest that the largest relative CBV changes occur near the top of our sub-volume (100 to 400 μm). This result may be confounded by the exponential attenuation of fluorescent signal as a function of depth; however, this effect was counteracted by the linear increase in laser power with depth into the sub-volume. The heterogeneous response from the microvasculature leads to a net gain in volume in quick and mid-latency responding vessels; but a net loss in volume in slowly responding vessels. This result again suggests late contraction of vertices that dilated at an earlier time point, ie. post-stimulus undershoot in the arterioles. Further, while relative volume changes (whether positive or negative) are larger in smaller vessels, the absolute volume contribution is dominated by larger vessels, in accordance with (Fernández-Klett et al, 2010).

Mapping the behaviour of the brain’s 3D microvascular network will facilitate development of quantitative models describing the hemodynamic adjustments underlying functional hyperemia. Such a detailed quantitative account of the microvascular network reactivity will further our understanding of the hemodynamically weighted responses measured in human neuro imaging - most notably BOLD fMRI - as well as provide a platform for future pre-clinical investigations of neurodegenerative disease models where neurovascular coupling is perturbed.
4.2 Summary of Major Findings

We measured:
- the resting primary somatosensory cortex luminal CBV (excluding pial surface and considering down to 600 μm in cortical depth) to be 3.4 ± 1.6 % (mean ± STD)
- radial dilation in response to Paradigm 2 of quick, mid-latency, and slow vertices to be 1.2 %, 1.4 %, and 2.0 %, respectively
- radial constriction in response to Paradigm 2 of quick, mid-latency, and slow vertices to be 1.3 %, 1.2 %, and 1.1 %, respectively
- upward propagation of dilation and constriction
- a sluggish response of constriction with respect to dilation in the latter phase of the vascular response
- net gain in total CBV for quick and mid-latency time window; net loss in total CBV for slow HRF time window

4.3 Future Work

An interesting extension of the current work would involve analysis of the spatial relationship between dilating and constricting regions. This investigation would allow mapping of the vascular steal or the shift of the blood pool from neuronally dormant to neuronally active areas. Furthermore, an in-depth analysis of the post-stimulus undershoot would contribute to the interpretation of BOLD fMRI data.

In future acquisition of similar data, standardization of the FOV with respect to the epicenter of neuronal response measured via EEG would be desirable, though such FOV placement would
still be variable with respect to microvascular anatomy. Also, imaging several FOVs with slightly overlapping regions and stitching them together post-imaging would give a much more comprehensive view of the microvascular response to stimulation. This strategy is, however, limited by the quality of the craniotomy and even more so by the total experimental time the animal must be kept anesthetized. As it was implemented in this study, imaging required approximately 1.5 hours. Even just doubling acquisition time (and therefore doubling the lateral FOV) would certainly compromise the physiology of the animal and render interpretation of results extremely difficult.
REFERENCES


[www.bme.arizona.edu/bme630/Lecture Public/6 Tissue Optics.pdf](http://www.bme.arizona.edu/bme630/Lecture Public/6 Tissue Optics.pdf)
APPENDIX

This appendix presents a comprehensive list of all significant results from a linear mixed effects model (Baayen et al., 2008). We probed for the effect of resting vascular parameters (resting radius, vertex cortical depth, and HRF type) on absolute and relative radius changes. The results of vertex-wise radius changes, vessel-wise radius changes, and vessel-wise volume changes are presented. All comparisons at \( p < 0.05 \).

**Protocol 1 Summary**

**Vertex wise DILATION**
1. radius percent and absolute difference depends on rest radius (negative) \{small vertices have larger relative and absolute dilations\}
2. radius percent (NOT absolute) difference depends on vertex depth (positive) \{dilations get larger as you go deeper\}

**Vertex wise CONstriction**
1. radius percent and absolute difference depends on rest radius (positive) \{small vessels have large relative and absolute constrictions\}
2. radius percent (NOT absolute) difference depends on vertex depth (negative) \{larger constrictions occur deeper\}

**Vessel wise DILATION**
1. vessel radius percent and absolute difference depends on rest radius (negative) \{smaller vessels have larger relative and absolute dilations\}
2. vessel radius percent or absolute difference does NOT depend on vessel depth

**Vessel wise CONstriction**
1. vessel radius percent and absolute difference depends on rest radius (positive) \{small vessels have large relative and absolute constrictions\}
2. vessel radius percent or absolute difference does NOT depend on vessel depth

**Vessel wise volume DILATION**
1. Nothing is significant!

**Vessel wise volume CONstriction**
1. volume absolute difference is dependent on rest radius (negative) \{larger vessels lose more absolute volume\}
Protocol 2 Summary

Vertex wise DILATION
1. radius percent and absolute difference depends (negatively) on rest radius (p < 0.05) {small vertices dilate more than large vertices}
2. percent and absolute difference depends on vertex depth (negatively) {larger dilation near the surface}
3. radius percent and absolute difference depends (positively) on HRF type (S7 only){percent difference of S7 is greater than Q1} **
4. vertex depth depends (negatively) on response type {vertex depth decreases from Q1 to M3/ S7 - ie. M3/S7 dilations are shallower than Q1}

Vertex wise CONSTRICIONS
1. radius percent and absolute difference depends on rest radius (positively) (p < 0.05) {small vertices contract more than large vertices}
2. radius percent and absolute difference depends on vertex depth (positively) {larger contractions near the surface}
3. radius percent difference is dependent (positively) on HRF type (only S7) {percent difference of S7 is smaller than Q1}
4. vertex depth depends (negatively) on response type {vertex depth decreases from Q1 to M3 - ie. M3 contraction is shallower than Q1}

Vessel wise DILATION
1. vessel percent and absolute difference is dependent on rest radius (negatively) {small vessels dilate more than large vessels}
3. vessel percent difference is dependent on HRF type (negatively) (S7 only) {percent difference of Q1 is greater than S7} **

Vessel wise CONSTRICIONS
1. vessel percent and absolute difference is dependent on rest radius (positive) {small vessels constrict more than large ones}
3. vessel percent difference is dependent on HRF type (positively) (only S7) {percent difference of S7 is smaller than Q1}

Vessel wise volume DILATION
1. vessel volume percent change is dependent on rest radius (negative) {smaller vessels gain lots of volume relatively}
2. vessel volume absolute difference is dependent on rest radius (positive) {larger vessels have larger absolute gains}

Vessel wise volume CONSTRICION
1. vessel volume percent change is dependent on rest radius (positive) {smaller vessels lose lots of volume relatively}
2. vessel volume absolute difference depends on rest radius (negative) {larger vessels lose more absolute volume}
** There is heterogeneity in vertex-wise latencies within vessels.

**Protocol 3 Summary**

Vertex wise DILATION
1. radius percent and absolute difference depends on rest radius (negative) {smaller vertices dilate more than large ones}
2. radius absolute difference depends on vertex depth (negative) {superficial vertices have larger dilations}
3. radius percent and absolute difference depends on HRF type (negative) {larger dilations are in Q1 than M3}

Vertex wise CONSTRICTION
1. radius percent and absolute difference depends on rest radius (positive) {smaller vertices have larger constrictions}
2. radius percent and absolute difference depends on vertex depth (positive) {superficial vertices have larger constrictions}

Vessel wise DILATION
1. radius percent and absolute difference depends on rest radius (negative) {smaller vessels dilate more than large ones}

Vessel wise CONSTRICTION
1. radius percent (not absolute) difference depends on rest radius (positive) {smaller vessels constrict more}

Volume DILATION
1. vessel volume percent difference is dependent on rest radius (negative) {smaller vessels gain more relative volume}
2. vessel volume absolute difference is dependent on rest radius (positive) {larger vessels gain more absolute volume}