MUTLI-SCALE FUNCTIONAL IMAGING OF CEREBROVASCULAR DYNAMICS WITH APPLICATION TO BRAIN TUMORS

by
Darian H. Hadjiabadi

A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science in Engineering

Baltimore, Maryland
August, 2017

© 2017 Darian H. Hadjiabadi
All Rights Reserved
Abstract

To meet cerebral metabolic demands, a sophisticated neurovascular mechanism is responsible for coupling neuronal activation with modulation of vascular tone and local cerebral blood flow (CBF). Due to this coupling, imaging techniques such as functional magnetic resonance imaging (fMRI) and optical imaging can characterize cerebrovascular dynamics as a surrogate to neural activation, making them useful tools for investigating brain functionality. These techniques have sparked widespread research into approaches capable of early detection of brain tumor-induced alterations in brain function, a consequence that often preordains cognitive decline. However, the complex interplay between abnormal brain tumor vasculature and disease-induced neurovascular uncoupling (NVU) can confound the interpretation of optical and MRI-derived functional imaging data. Therefore, in this thesis we sought to elucidate the effects of brain tumor progression on neuronal function through multi-scale analysis of resting state cerebrovascular dynamics. We first quantified brain tumor-induced changes on the resting state fMRI (rsfMRI) signal relative to that from healthy murine brains. We observed that brain tumors induced brain-wide reorganization of resting state networks extending to the contralateral hemisphere, accompanied by global attenuation of blood-oxygen-level-dependent (BOLD) signal fluctuations. Histological validation suggested that these connectivity alterations may be attributable to NVU. Next, we employed laser speckle contrast imaging (LSCI) and optical intrinsic signal (OIS) imaging to acquire multi-contrast hemodynamic maps of the entire murine cortical surface during both, the resting state and in response to a vasodilatory challenge. By
exploiting the high temporal resolution and microvascular-scale spatial resolution, we characterized these changes at a level not possible with conventional fMRI techniques. Finally, we examined stimulus-induced changes in neurovascular dynamics using multi-contrast optical imaging. Using the neuronal and hemodynamic information acquired from the murine auditory cortex in response to auditory stimulation, we utilized two mathematical models to isolate regions exhibiting a frequency specific vascular response. Collectively, our findings on brain tumor induced alterations in resting state connectivity using multi-scale functional imaging methods lay the foundation for developing a novel biomarker for brain cancer progression. Furthermore, our multimodality research brings to the forefront the growing importance of functional imaging in neuroscience and beyond.

**Thesis Committee**

<table>
<thead>
<tr>
<th>Arvind P. Pathak, Ph.D.</th>
<th>Nitish V. Thakor, Ph.D.</th>
<th>Betty M. Tyler, Ph.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>The Russel H. Morgan Department of Radiology &amp; Radiology Science / Department of Biomedical Engineering</em></td>
<td><em>Department of Biomedical Engineering</em></td>
<td><em>Department of Neurosurgery</em></td>
</tr>
<tr>
<td>Johns Hopkins University</td>
<td>Johns Hopkins University</td>
<td>Johns Hopkins University</td>
</tr>
<tr>
<td><a href="mailto:pathak@mri.jhu.edu">pathak@mri.jhu.edu</a></td>
<td><a href="mailto:thakorjhu@gmail.com">thakorjhu@gmail.com</a></td>
<td><a href="mailto:btyler@jhmi.edu">btyler@jhmi.edu</a></td>
</tr>
</tbody>
</table>
I would like to express gratitude towards the committee for taking their time to provide critical feedback on this work. To Drs. Betty Tyler and Nitish Thakor, I thank the both of you for our collaborations together in many different projects. I would also like to thank my mentor, Dr. Arvind Pathak, for the many conversations which we have had, for your objective and critical feedback on all things science, and for your infinite patience. We learned a lot together.

In addition, it was wonderful to have spent time amongst the lab’s many unique and humble personalities. To Janaka Senarathna, it was a pleasure to discuss ideas with you, even if mine were usually not very good. I will miss your near perfect “Holmesian” logic. To Stacy Gil, the mice in this lab will not be the same without out. Thank you for all your help in animal preparations and experimentation. To Victoria Fang and Julia Szewc, thank you for your help in the brain clearing procedure. To Akanksha Bhargava, Gayatri Susarla, Namrata Batra, and Callie Deng, remember to floss (and also do amazing things).

Last but not least, I am grateful to have a family that has supported my desire to pursue graduate school.
# Table of Contents

CHAPTER 1: An overview of neurovascular coupling in health and disease

1.1 Introduction ........................................................................................................ 1
1.2 The Neurovascular Unit in Homeostasis ................................................... 3
1.3 The Vasculature of Brain Tumors ................................................................. 7
1.4 Brain Tumors Alter the Neurovascular Unit ............................................. 11
1.5 A Survey of Non-function MRI Techniques for Monitoring Tumor Progression ................................................................. 12
1.6 Functional MRI of Brain Tumors ................................................................. 13

CHAPTER 2: A function MRI (fMRI) study demonstrating that brain tumors disrupt the ‘resting-state’ connectivity between brain regions

2.1 Introduction ........................................................................................................... 17
2.2 Methods ................................................................................................................ 20
   2.2.1 Animal Preparation ...................................................................................... 20
   2.2.2 In Vivo MRI Protocol .................................................................................. 21
   2.3.3 Histology and Immunofluorescence Protocol ............................................ 21
   2.3.4 Image Processing ......................................................................................... 22
2.3 Results .................................................................................................................. 25
   2.3.1 Visualization of brain tumor-induced alterations in resting state connectivity ......................................................................................................................... 25
   2.3.2 Brain tumors disrupt inter – and intra-hemispheric resting state connectivity .......................................................................................................................... 27
   2.3.3 Brain tumor progression (i.e. increasing tumor volume) alters resting-state connectivity .................................................................................................................. 30
   2.3.4 Brain tumors attenuate the resting state BOLD signal across brain regions ................................................................................................................................. 32
2.3.5 Converse correlation analysis and generation of “tumor connectivity” maps.................................................................33
2.3.6 Brain tumors disrupt the neurovascular unit..............................35

2.4 Discussion.........................................................................................38

CHAPTER 3: Exploring cerebrovascular dynamics in brain tumor bearing brains using multi-contrast optical imaging...............47

3.1 Introduction..................................................................................47

3.2 Methods.........................................................................................50
  3.2.1 Animal Preparation..................................................................50
  3.2.2 In Vivo Imaging protocol with Intact Skull................................. 50
  3.2.3 Assessing brain tumor growth..................................................52
  3.2.4 Imaging preprocessing...............................................................54
  3.2.5 Registration and Region of Interest Segmentation......................55
  3.2.5 Data Analysis...........................................................................56

3.3 Results.............................................................................................61
  3.3.1 Benchtop optical imaging system was able to capture microvascular resolution images of the whole-murine brain......................61
  3.3.2 Registration of murine 2D neocortex atlas to mouse subject and region of interest segmentation..................................................62
  3.3.3 Visualization of brain tumor-induced alterations in resting state connectivity – initial findings.................................................63
  3.3.4 Whole-brain disruptions of resting state connectivity induced by tumor – initial findings.........................................................65
  3.3.5 Temporal-frequency analysis reveals temporal instability in tumor region compared to contralateral hemisphere – initial findings........66
  3.3.6 Entropy metric appears to be a useful tool for isolating tumor affected tissue – initial findings...................................................69
  3.3.7 Tracer kinetic modeling enables arteriole and venule separation...71
CHAPTER 4: Additional applications of functional optical imaging

4.1 Introduction .................................................................................................................. 80
4.2 Methods ..................................................................................................................... 83
   4.2.1 Cranial window preparations ................................................................................. 83
   4.2.2 Experimental Procedure ...................................................................................... 84
   4.2.3 Image Processing ................................................................................................. 85
4.3 Results ....................................................................................................................... 90
   4.3.1 Microscope is capable of acquiring microvascular-scale optical images .............. 90
   4.3.2 Multi-modal optical acquisition of neurovascular dynamics to auditory stimulation.............................................................................................................................. 90
   4.3.3 Utilizing frequency specific neuronal ‘hotspots’ for wide-area functional mapping of vascular activation ................................................................. 92
   4.3.4 Wide-field mapping of the hemodynamic response function yields further insight on neurovascular dynamics ................................................................. 95
   4.3.5 Modeling the hemodynamic response function may overall have limited utility .............................................................................................................................. 98
4.4 Discussion .................................................................................................................. 99

CHAPTER 5: Future Directions ......................................................................................... 103

BIBLIOGRAPHY ............................................................................................................. 106

CURRICULUM VITAE ....................................................................................................... 120
List of tables

Table 3.1: ScaleS experimental timeline..........................................................................................53

Table 4.1: Spatial heterogeneity in hemodynamic response function manifesting from 4 kHz auditory stimulation ........................................................................................................98
List of figures

Figure 1.1: A schematic of the healthy neurovascular unit.................................5

Figure 1.2: Tumors disrupt the neurovascular unit...........................................6

Figure 1.3: The endothelium is a key player in neurovascular coupling.................7

Figure 1.4: Brain tumor vasculature is highly divergent from contralateral
hemisphere........................................................................................................10

Figure 1.5: CVR maps provide evidence of NVU in tumor-affected brain region......15

Figure 1.6: NVU confounds interpretation of task-based and resting-state fMRI........16

Figure 2.1: Visualization of alterations in resting-state connectivity induced by a medium
size brain tumor in the striatum.........................................................................26

Figure 2.2: Brain tumors disrupt inter- and intra-hemisphere resting state functional
connectivity........................................................................................................29

Figure 2.3: Box and whisker plots of correlation coefficients between ROI pairs
computed for normal and tumor-bearing brains..................................................30

Figure 2.4: Changes in resting state connectivity as a function of brain tumor
progression........................................................................................................31

Figure 2.5: Tumors suppress BOLD fluctuations across brain regions..................33

Figure 2.6: Reverse analysis of “tumor connectivity” shows that the small tumor is
synchronized with intrahemispheric ROIs and larger tumor synchronized with
interhemispheric ROIs..........................................................................................34

Figure 2.7: The neurovascular unit is disrupted in brain tumors.........................37

Figure 3.1: Benchtop optical imaging system was able to acquire microvascular
resolution images of the whole-murine brain..................................................62
Figure 3.2: Registration of murine 2D neocortex atlas to mouse subject and region of interest segmentation

Figure 3.3: Visualization of brain tumor-induced alterations in resting state connectivity

Figure 3.4: Whole-brain disruptions of resting state connectivity induced by tumor – initial findings

Figure 3.5: Temporal-frequency analysis reveals temporal instability in tumor region compared to contralateral hemisphere – initial findings

Figure 3.6: Entropy metric appears to be a useful tool for isolating tumor affected tissue – initial findings

Figure 3.7: Tracer kinetic modeling enables arteriole and venule separation

Figure 4.1: Microscope was capable of acquiring microvascular level optical images

Figure 4.2: Multi-modal optical acquisition of neurovascular dynamics to auditory stimulation

Figure 4.3: Utilizing frequency specific neuronal ‘hotspots’ for wide area functional mapping of vascular activation

Figure 4.4: Wide-field mapping of the hemodynamic response function yields further insight on neurovascular dynamics

Figure 4.5: Modeling the hemodynamic response function may have limited utility
Chapter 1: An Overview of Neurovascular Coupling in Health and Disease

This chapter is adapted from a review article (Pak et al., 2017) that we recently published in the Journal of Cerebral Blood Flow & Metabolism, and its contents have been excerpted in accordance with the publisher’s ‘Green Open Access’ policy (https://us.sagepub.com/en-us/nam/journal-author-archiving-policies-and-re-use).

DOI: 10.1177/0271678X17707398

1.1 Introduction

The neurovascular coupling mechanism is responsible for mediating cerebral blood flow (CBF) with neural firing to meet fluctuating cerebral energy demand, which also constitutes the basis of functional magnetic resonance imaging MRI (fMRI) (Metea and Newman, 2006; Roy and Sherrington, 1890). Specifically, neurogenic induced vasodilation leads to an overall net influx of oxyhemoglobin, reducing the heterogeneity of local magnetic fields due to its paramagnetic properties. This is the premise of the blood-oxygen-level-dependent (BOLD) contrast mechanism underlying fMRI (Ogawa et al., 1990). However, not all changes in the BOLD signal are neurogenic – it is a mixture of several phenomena including changes in cerebral blood flow (CBF), cerebral blood volume (CBV), and the cerebral metabolic rate of oxygen consumption (CMRO$_2$) (Hoge et al., 1999). Nonetheless, it is the neurovascular coupling mechanism which has given fMRI a significant role in interrogating brain functionality (Logothetis and Wandell, 2004).
Functional mapping of brain regions using fMRI has been done in both the presence (task-based; tb-fMRI) and in the absence of a task (i.e. resting-state; rs-fMRI). Tb-fMRI allows researchers to capture neurovascular coupling mediated increases in the BOLD signal. In such cases, studies have demonstrated that after task performance there is a latent period of ~1-2 seconds followed by a ~ 4% rise in BOLD signal (Bandettini et al., 1992). Recently, it has been suggested that brain regions which exhibit temporally correlated BOLD fluctuations in a low frequency band (0.01 – 0.1 Hz; acquired during the ‘resting-state’) also yields important information regarding brain functionality (Biswal et al., 1995). This concept has been used to map resting-state brain connectivity (Biswal et al., 1995) and forms the basis of rs-fMRI. However, clinical and preclinical studies have demonstrated that brain tumors can exhibit neurovascular uncoupling (NVU) that can confound the interpretation of brain activation in tb-fMRI (Ulmer et al., 2003) and that of resting-state connectivity patterns in rs-fMRI (Agarwal et al., 2016b). Therefore, understanding how the neurovasculature is affected by diseases (e.g. brain tumors and stroke) serves as an important first step to utilizing the fMRI signal as a disease biomarker.

Here, we review the physiology of the neurovascular unit and how it is functionally altered by brain cancer cells. We first discuss the components of the neurovascular unit. Next, we look at the vasculature of brain tumors and illustrate how NVU may be induced. Thirdly, we investigate how NVU can affect MRI data interpretation, closing with a brief overview of advances in functional imaging methods to permit the clinical evaluation of brain tumors with NVU.
1.2 The neurovascular unit in homeostasis

The complex relationship between the neuron and the vasculature has been a topic of research going back to the 19th century. We owe much of our understanding of the neurovascular unit, the fundamental unit underlying neurovascular coupling, to pioneers such as Roy and Sherrington, and Ramon y Cajal. Roy and Sherrington discovered that stimulating the medulla oblongata induced vasoconstriction in other organs, increased arterial pressure, bringing blood to the stimulated region (Roy and Sherrington, 1890). Ramon y Cajal, on the other hand, emphasized that astrocytes were more than just structural support cells (Araque et al., 2001). Ramon y Cajal’s insulation theory hypothesized that astrocytes served as barriers between neighboring neurons (Navarrete and Araque, 2014), while research since then has updated this theory to suggest that astrocytes play a critical role in the communication between cerebral blood vessels and neurons (Iadecola and Nedergaard, 2007).

Currently, we are aware of three fundamental elements that comprise the neurovascular unit: astrocytes, pericytes, and endothelial cells (Fig 1.1). Astrocytes play multiple roles in maintaining homeostatic brain functionality. Firstly, astrocytes provide a medium for which neurotransmitters released from activated neurons can be transmitted to the vasculature. Specifically, the astrocytic end feet have been shown to play a significant role in facilitating neurovascular coupling (Kimelberg and Nedergaard, 2010) due to its intimate contact with blood vessels (Figs 1.1, 1.2a). When the signal cascade reaches the endfeet, vasoactive molecules, which regulates the tone of vascular
smooth muscle cells (VSMCs), are released and contribute to vasodilation (Watkins et al., 2014). Additionally, astrocytes can produce arachidonic acid (AA), known to cause vasoconstriction (Huneau et al., 2015), and are responsible for maintaining the blood brain barrier (BBB) through their structural support of tight junctions (Abbott et al., 2010). Disruption of these tight junctions allows for pathogens to enter the brain parenchyma, as if often observed in patients with infections or brain cancer (Lee et al., 2009).

Pericytes also contribute to healthy neurovascular coupling (Winkler et al., 2011). During development, pericytes are thought to play a role in the formation of the BBB (Watkins et al., 2014) and promote the maturation of blood vessels (Daneman et al., 2010). This is done through a signaling pathway which inhibits endothelial cell proliferation (Daneman et al., 2010).

While astrocytes and pericytes were thought to be the primary mediators of neurovascular coupling, recent evidence has suggested that vascular endothelial cells may also play an important role (Chen et al., 2014). Chen et. al observed that disruption of endothelial cells membranes via reactive oxygen species (ROS) (Howitt et al., 2013) prevents downstream endothelial cell signaling (Chen et al., 2014). Namely, when ROS were not present, retrograde dilation of pial artery beds in response to hyperemia was observed, while, in contrast, the presence of ROS blocked such dilations (Chen et al., 2014) (Fig 1.3). Collectively, this experiment has suggested that disruption of endothelial cell membranes may lead to NVU.
Intensive research has brought to the forefront additional potential players in neurovascular coupling. The ‘glymphatic’ system, a brain-wide paravascular pathway for clearing extracellular proteins (Iliff et al., 2012), has been shown to operate via glial water flux and plays a role akin to the lymphatics system. However, this system's role in NVU remains to be observed. Additionally, interneurons have been shown to release vasoactive molecules responsible for both vasodilation and vasoconstriction (Cauli et al., 2004). It is unclear as to the extent to which this mechanism affects neurovascular coupling, with conservative estimates claiming that interneurons may simply fine tune local hemodynamics (Attwell and Iadecola, 2002).
**Figure 1.1: A schematic of the healthy neurovascular unit**

Neurovascular homeostasis and overall maintenance of the BBB is maintained through a complex interplay of central and peripheral cells. Here, we observe that astrocytes play a central role in transmitting information from neuron to vessel. Astrocytes maintain intimate contact with the endothelium via the astrocytic end feet. In addition, pericytes are in direct contact with endothelial cells, where crosstalk is initiated by peg-socket junctions. Ongoing research is dedicated to finding additional key players of the neurovascular unit. Adapted with permission (Obermeier et al., 2013).

![Image of healthy neurovascular unit](image1)

**Figure 1.2: Tumors disrupt the neurovascular unit**

Immunofluorescent labeling and histological acquisition of the neurovascular unit in (a) non-tumor tissue shows robust astrocytic-vascular coverage indicating a healthy neurovascular unit. In contrast, (b) histology of tumor tissue from 9L brain tumor bearing mouse shows a dearth of astrocytic coverage and displaced astrocytes endfeet. All images were acquired at 20x magnification. Here, DAPI (blue channel) labeled cell nuclei, GFAP (green channel) labeled astrocytes, and blood vessels were perfused with DEX-TRITC (red channel).
**Figure 1.3: The endothelium is a key player in neurovascular coupling**

Vascular perfusion levels, as measured through total hemoglobin, before and after the presence of endothelial cell damaging ROS species induced through light-dye treatment. (a) Vascular topology acquired from 534nm illumination and resulting (b) map of vascular perfusion (no treatment). (c) In the presence of ROS species, the absence of dilation in pial arteries is evident when compared to (a). This observation suggests the endothelium plays an important role in neurovascular coupling. Adapted with permission (Chen et al., 2014).

### 1.3 The vasculature of brain tumors

Vascular phenotyping analysis conducted by Kim et al has highlighted just how disparate brain tumor vasculature is compared to that in the contralateral brain hemisphere (**Fig 1.4**) (Kim et al., 2011). Such abnormalities arise through a complex interplay involving tumor cell proliferation and the imbalance in angiogenic growth factors [Jain, Nat reviews neurosci]. Glioma growth often begins through the mechanism of co-option, in which cells grow around pre-existing brain vasculature (Holash et al., 1999). This results in the invasion of perivascular space and the development of satellite tumors (Folkman, 1971; Holash et al., 1999). However, asis often the case where the nascent tumor mass may not necessarily have the proper oxygen supply to meet energy
demands, and therefore apoptosis is initiated. Apoptosis leads to the release of vascular endothelial growth factor (VEGF), a molecule that drives angiogenesis (Holash et al., 1999). Furthermore, overall poor brain tumor perfusion leads to a hypoxic tumor microenvironment, the result of which is the increased deployment of angiopoietin-2 (ANG-2). Collectively, the combination of VEGF and ANG-2 steers the formation of new vessels and migration and proliferation of endothelial cells (Zagzag et al., 2000). In addition, hypoxia and increased ANG-2 concentrations can lead to increased levels of hypoxia-inducible factors (HIFs), which further upregulates VEGF (Hardee and Zagzag, 2012). This increase in VEGF causes increased levels of brain-derived neurotrophic factor (BDNF), which is responsible for vascular development, maintenance of the cerebrovascular endothelium, and the interplay between angiogenesis and neurogenesis (Hardee and Zagzag, 2012; Kim et al., 2004; Louissaint et al., 2002).

Normally, ANG-2 and angiopoetin-1 (ANG-1) act as antagonists. However, increased concentrations of ANG-2 in the tumor microenvironment results in elevated suppression of ANG-1, a molecule which has been shown to recruit pericytes (Reiss et al., 2005), and leads to compromise of blood vessel integrity (Daneman et al., 2010). Furthermore, the binding of ANG-2 to its TIE-2 receptor can lead to disruption of endothelial cell junctions, compromising the BBB (Hardee and Zagzag, 2012; Reiss et al., 2005). As a result, complications such as vasogenic edema (Jain et al., 2007a) arise and can distort the homeostatic interstitial fluid pressure (IFP) (Boucher et al., 1997; Jain et al., 2007b) via fluid leakage into the brain. This phenomenon can complicate drug delivery (Jain, 1994). Furthermore, edema has been shown to occur along white matter
tracts (Monajati and Heggeness, 1982), eluding to possible disruptions of connectivity between brain regions.

There are two additional methods in which glioma cells may grow: vasculogenesis and intussusception. Vasculogenesis arises through the recruitment of bone-marrow derived cells (BMDC), which are incorporated into new vasculature (Du et al., 2008; Hardee and Zagzag, 2012). While its frequency in glioblastoma (GBM) is still being debated, it has the potential to contribute to anti-angiogenic therapy resistance and progression of GBM (Jhaveri et al., 2016; Liebelt et al., 2016). ‘Intussusception’ angiogenesis (Nagy et al., 2010) arises through the physical disruption of preexisting vascular lumen as a result of protruding interstitial tissue columns (Patan et al., 2001). This results in the remodeling of the cerebrovasculature. We will now see how these GBM induced physical and chemical alterations can affect functional and structural integrity of the neurovascular unit.
Figure 1.4: Brain tumor vasculature is highly divergent from contralateral hemisphere

Box and whisker plots of two vascular and two diffusion parameters for D12 contralateral, D12 tumor, D17 contralateral, and D17 tumor ROIs (n=5 for all groups, *P<0.05). (A) The median vessel length, (B) average vessel radius, (C) MVD, (D) $L_v$, (E) FV, (F) median vessel tortuosity (G) ADC, and (H) FA. ADC, apparent diffusion coefficient; D12, post-inoculation day 12; D17, post-inoculation day 17; FA, fractional anisotropy; FV, fractional vascular volume; $L_v$, length per unit volume; MVD, microvessel density; ROI, region of interest. Adapted with permission (Kim et al., 2011).
1.4 Brain tumors alter the neurovascular unit

Converging evidence has shown that NVU manifests across a wide spectrum of diseases (Stanimirovic and Friedman, 2012), including stroke, Alzheimer’s, Parkinson’s, and brain tumors (Agarwal et al., 2016a). Histopathological research in brain tumors has shown that gliomas can invade perivascular space and disrupt the various elements of the neurovascular units (Lee et al., 2009). While astrocytic spatial distribution in healthy tissue has been shown to be localized around arteries and capillaries (Watkins et al., 2014) (Fig 1.2a), in contrast Fig 1.2b shows that astrocytes can dissociate as a result of glioma cell invasion. Additionally, glioma cells have been shown to take over surrounding blood vessels, favoring small capillaries (Watkins et al., 2014). These effects taken together prevent vasoactive molecules from reaching the endothelial cell wall, leading to attenuation of the cerebrovascular response and overall alterations to neurovascular coupling.

It has also been suggested that glioma cells are capable of ‘hijacking’ vascular tone (Watkins et al., 2014). Here, glioma cells utilize their increased expression of Ca²⁺-activated K⁺ channels to alter K⁺ concentration, which in turn controls blood vessel diameter (Watkins et al., 2014). This gives rise to the notion that even a single glioma cell can induce NVU.

Growth of glioma cells on the abluminal surface of the cerebrovasculature has additional consequences. It has already been mentioned that disruption of endothelial cells may result in NVU through attenuation downstream endothelial cell signaling
Furthermore, the combination of down regulation of tight conjunction proteins (claudin-1,-3, and -5) and displacement of pericytes (Stanimirovic and Friedman, 2012) may result in disruption of the BBB (Liebner et al., 2000). This result leads to permeable blood vessels, activates pro-inflammatory responses (Liebner et al., 2000), and, collectively, NVU.

1.5 A survey of non-functional MRI techniques for monitoring tumor progression

Quantification of angiogenesis-induced vascular remodeling has been made possible (Emblem et al., 2014; Pathak et al., 2008) by exploiting the kinetics of contrast agents as they transit through the cerebrovasculature (Tofts et al., 1999). Examples of approaches to this effect include dynamic contrast-enhanced (DCE) and dynamic susceptibility contrast (DSC) MRI (Choyke et al., 2003; Quarles et al., 2012). Hemodynamic variables including CBV, CBF, and mean transit time (MTT) can be acquired using T2* DSC perfusion imaging (Ostergaard et al., 1996; Rosen et al., 1991; Weisskoff et al., 1993), while T1 steady state DCE can be used to measure permeability. Collectively, these variables facilitate monitoring of tumor angiogenic status and breaches of the BBB (Tofts and Kermode, 1991).

Development of endogenous contrast-based MRI techniques has also played an instrumental role in brain tumor monitoring. One such technique, arterial spin labeling (ASL), allows for perfusion quantification by using magnetically tagged arterial blood water protons as an intrinsic tracer. This method has been employed to characterize
brain tumor-induced changes in the neurovasculature (Moffat et al., 2005; Pollock et al., 2009), where it has been found especially suitable for assessing CBF. Unfortunately, these above-mentioned MRI techniques are unable to effectively detect vessel co-option, which does not involve hyperpermeable tumor vessels (Folkman, 1971; Holash et al., 1999).

### 1.6 Functional MRI of brain tumors

BOLD fMRI is playing a major role in functional brain research. Neurosurgeons quite often rely on tb-fMRI to map eloquent cortical regions during surgery; however, mounting evidence suggests that rs-fMRI may serve as an alternative approach. Since fMRI is used as a surrogate for neural activation through measurement of CBF fluctuations, tumor-induced NVU has the potential to confound BOLD response when mapping crucial areas (Agarwal et al., 2016a; Holodny et al., 1999; Zaca et al., 2014). Additionally, research has shown that NVU is possible in high grade (Hou et al., 2006), intermediate grade (Pillai and Zaca, 2011), and low grade (Pillai and Zaca, 2011) gliomas. For example, Hou et al. presented increased CBV as a result of neovascularization, and NVU induced BOLD signal attenuation in enhancing portions of grade IV GBM (Hou et al., 2006). These findings lead to the realization that attenuation of the BOLD signal is caused by two sources that are not easy to distinguish - NVU and lack of neural response as GBMs progress (Zaca et al., 2014).

To address this issue, cerebrovascular reactivity (CVR) mapping during hypercapnia induction via breath-holding (BH CVR) in conjunction with tb-fMRI has been
proposed (Pillai and Mikulis, 2015). The BH CVR approach provides an opportunity to assess CBF and vasodilation in the absence of task-induced neural stimulation (Pillai and Zaca, 2012). Patients with low grade tumor who performed BH CVR and tb-fMRI mapping displayed impaired sensorimotor activation in brain region ipsilateral to tumor location (Zaca et al., 2014). In contrast, contralateral sensorimotor regions exhibited normal CVR (Fig 1.5) (Zaca et al., 2014). Attenuation of BOLD response and impaired CVR constrained to ipsilateral brain region, and a lack of prominent neurological deficits indicates that NVU did not go beyond ipsilateral cortex. Additionally, rs-fMRI mappings have shown similar results collectively suggesting that NVU can pose a problem for both tb and rs-fMRI (Agarwal et al., 2016a; Agarwal et al., 2016b) (Fig 1.6). In order to understand how the fMRI BOLD signal is perturbed in the presence of tumor, a complete understanding of the interplay between tumor vasculature, NVU, and the BOLD contrast mechanism is needed. Proper considerations of these factors may allow for the BOLD signal to become powerful tool for monitoring both angiogenic and co-optive tumor phenotypes.

NVU induced loss of structural connectivity is also quite evident during glioma progression, as has been shown with diffusion tensor imaging (DTI) (Lu et al., 2003; Pathak et al., 2011). Structural connectivity can be investigated with numerous additional diffusion methods such as diffusion spectrum imaging (DSI), diffusion kurtosis imaging (DKI), and high angular resolution diffusion imaging (HARDI). Together, the complementary role of structural connectivity to functional connectivity will need to be investigated in greater detail.
Collectively, the numerous MRI platforms at our disposal provides an opportunity to facilitate monitoring of GBM progression independent of phenotype, and to segregate the effects of NVU from the absence of neural activity.

**Figure 1.5: CVR maps provide evidence of NVU in tumor-affected brain region**

CVR maps overlaid on T2 FLAIR images obtained on a 7T MRI system during breath hold task. Patient shown here was afflicted with low-grade oligoastrocytoma (WHO grade II). As shown by the white arrow, one can see abnormally decreased and absent CVR in tumor vicinity relative to contralateral hemisphere. This is an indication of NVU. All CVR maps were thresholded at a z-score > 1.0.
Figure 1.6: NVU confounds interpretation of task-based and resting-state fMRI

(a) task-based and (b) resting-state fMRI maps from a patient with low-grade (WHO grade II) non-enhancing oligodendroglioma, overlaid on FLAIR T2 images. (a) Vertical tongue movement task activation map (blue arrow) at 3T thresholded at z-score > 4.5. (b) The resting-state fMRI map displaying sensorimotor activation (blue arrow) derived from ICA with order of 30, thresholded at z-score > 5.0. The white arrow points to the expected areas of sensorimotor cortex activation in all panels. Both task-based and resting-state fMRI methods demonstrated NVU.
Chapter 2: A Functional MRI (fMRI) Study Demonstrating that Brain Tumors Disrupt the ‘Resting-State’ Connectivity Between Brain Regions

This chapter is currently in submission as a full paper in the journal *NeuroImage*.


2.1 Introduction

The devastating consequence of a brain tumor on a patient’s quality of life has sparked widespread research into approaches capable of early detection of tumor-induced alterations in brain functions. Noninvasive techniques such as magnetoencephalography (MEG) (Bartolomei et al., 2006), and task-based fMRI have been at the forefront of such efforts (Holodny et al., 1999). However, MEG suffers from issues of spatial localization of the signal (Hillman, 2014) because one needs to solve the inverse problem of localizing neural activity within the brain from the induced magnetic fields measured outside it (Hämäläinen et al., 1993). In contrast, task-based fMRI has proven immensely useful for pre-surgical mapping of eloquent cortex prior to surgical resection. However, this method can be challenging to conduct on brain tumor patients due to issues such as task noncompliance, the constraint of long imaging times, and false-negatives (Zaca et al., 2014) arising from neurovascular uncoupling (Ulmer et al., 2003). Although one could circumvent the first two issues by using task-independent
resting-state fMRI (rsfMRI) approaches, little is known about how the blood-oxygen-level-dependent (BOLD) rsfMRI signal is modulated by the presence of a brain tumor. Therefore, the objectives of this preclinical study were to systematically quantify brain tumor-induced changes on the BOLD rsfMRI signal.

Resting-state functional MRI is based on the premise that distinct brain regions exhibit temporally correlated spontaneous fluctuations in blood flow to meet the energy demands necessary for healthy brain function (Biswal et al., 1995). As a result, rsfMRI has been successfully used to map changes in ‘connectivity’ of spatially distinct brain regions in an array of disease models using a stimulus-independent paradigm. These include mapping alterations in neuronal connectivity in patients with stroke (Golestani et al., 2013), schizophrenia (Lynall et al., 2010), bipolar disorder (Mamah et al., 2013), multiple sclerosis (Filippi et al., 2013), and Alzheimer’s disease (Agosta et al., 2012). Recent, work by Hillman and colleagues elegantly demonstrated that resting-state hemodynamics are spatiotemporally coupled to synchronized neural activity in excitatory neurons (Ma et al., 2016) thereby validating some of the biophysical mechanisms underpinning the rsfMRI signal. However, like task-based fMRI, rsfMRI studies in patients with brain tumors have been limited (Agarwal et al., 2016; Chow et al., 2016). This is attributable to several factors. The first is that the abnormal vascular architecture (Kim et al., 2011) and anomalous blood flow characteristics (Jain et al., 2007) modulates the BOLD signal lying within the tumor region (Chow et al., 2016). Next, there is the phenomenon of neurovascular uncoupling (NVU) in which brain tumor cells disrupt the homeostatic coupling between neurons and astrocytes and the
underlying mechanism responsible for cerebral blood flow modulation. This can confound the interpretation of resting-state connectivity in patients (Agarwal et al., 2016).

Early BOLD signal fluctuation-based MRI studies of cancer focused on elucidating the functional status or ‘maturity’ of the tumor vasculature in preclinical tumor models (Baudelet et al., 2006). More recent studies have employed spontaneous BOLD fluctuations to generate maps of ‘active’ tumor regions using independent component analysis, and showed that heterogeneous tumor vessel functionality can result in uniquely correlated regions (Goncalves et al., 2015). However, neither of these studies employed an orthotopic brain tumor model to investigate the relationship between the brain tumor microenvironment (TME) and the BOLD rsfMRI signal. While rsfMRI studies in rodents have only been reported recently, they have demonstrated that certain resting state networks can be observed across mammalian species (Pawela et al., 2008). For example, rsfMRI studies have shown that the functional connectivity in the limbic, motor, visual, and somatosensory networks can be successfully mapped in rodents (Bergonzi et al., 2015; Jonckers et al., 2011; White et al., 2011). Collectively, these developments afford us the opportunity to first elucidate the effect of brain tumors on resting state connectivity in rodent models and then potentially translate these findings to patients.

In this study, we characterized the effects of the abnormal brain tumor vasculature, anomalous tumor blood flow, and cancer cell-induced neurovascular
uncoupling on rsfMRI dynamics. Specifically, we aimed to quantify and compare resting-state BOLD signal dynamics in healthy murine brains relative to brain tumor-bearing murine brains. We examined tumor-induced alterations in the resting-state connectivity between multiple brain regions across both cerebral hemispheres. Furthermore, we demonstrated global tumor-induced modulations in resting-state BOLD signal fluctuations. The effect of brain tumor progression on resting-state connectivity between brain regions, as well as between the tumor and cortex were also investigated. Finally, we validated our in vivo rsfMRI findings with histological evidence to illustrate how neurovascular remodeling induced by the presence of brain tumor cells can profoundly modulate the BOLD rsfMRI signal.

2.2 Methods

2.2.1 Animal Preparation

9L-GFP brain tumor cells were orthotopically inoculated into the cortices of SCID mice (n=8) as described in (Pathak et al., 2001). Brains of healthy SCID mice (n=8) served as the control group. Briefly, severe combined immune deficient (SCID) mice weighing approximately 30g (Charles River/NCI, Frederick, MD), were anesthetized with a xylazine/ketamine cocktail for tumor cell inoculation. Their heads were immobilized and using an aseptic technique, a 1mm burr hole drilled in the skull 1mm anterior and 2mm lateral to the bregma on the right side. A 10 µl gas-tight syringe (Hamilton Comp, Reno, Nevada) was used to inject $10^5$ cells of the 9L-GFP gliosarcoma cell line, into the right frontal lobe at a depth of 3mm relative to the dural surface. The 9L cell line was
obtained from the Brain Tumor Biology Laboratory, and grown in DMEM (Gibco, Gaithesburg, MD) with 10% FBS. Cells were expanded immediately prior to inoculation. The injection time was 5 min, after which the needle was retracted slowly for an additional 5 min. The skin was then closed with surgical staples that were removed prior to MR imaging. All animal studies were performed according to institutional guidelines and the NIH “Guide for the Use and Care of Laboratory Animals”.

2.2.2 In Vivo MRI Protocol

All animals were imaged in vivo on a 400 MHz vertical bore Bruker spectrometer under isoflurane anesthesia (1-1.5% mixed with air and oxygen at a 3:1 ratio) using the following sequences after localized shimming: (i) T2w rapid acquisition with relaxation enhancement (RARE), RARE-factor=8, TE=15.0 ms, TR=3.5 s, NA=8, in-plane resolution=0.1 mm×0.1 mm, 16-24 coronal slices, slice thickness =0.3 mm; (ii) 4-segment gradient-echo EPI, TE=8.4 ms, TR= 400 ms, 110 repetitions, in-plane resolution=0.2 mm×0.2 mm, 16-24 coronal slices, slice thickness =0.3 mm. Animals were imaged under isoflurane anesthesia with body temperature maintained at 37°C and respiration rate at 40-60 bpm.

2.2.3 Histology and Immunofluorescence Protocol

After in vivo MRI, animals were perfused with the intravascular tracer dextran-TRITC (70 kDa, Zymed Laboratories, San Francisco, CA) via the tail vein, euthanized, brains were excised, fixed in 10% buffered formalin and frozen in liquid nitrogen for cryosectioning. Adjacent 12 µm frozen brain sections were cut onto silanized slides and
immunofluorescent labeling of the neurovascular unit components carried out. Astrocytes were labeled with anti-glial fibrillary acidic protein (GFAP) antibody (Cell Signaling, Danvers, MA), vasculature associated smooth muscle with α-smooth muscle actin antibody (Sigma-Aldrich, St. Louis, MO), and blood vessel endothelium was detected on the same tissue with anti-mouse laminin antibody (Sigma-Aldrich, St. Louis, MO). Slides were counterstained with DAPI (Molecular Probes Inc., Eugene, OR) and cover-slipped. Slides were imaged on a Nikon ECLIPSE-TS100 microscope (Nikon Instruments Inc., NY) with the appropriate filters for detecting immunofluorescence. Regions-of-interest (ROIs) were digitized at 20× and 40× using a SPOT INSGHT™ CCD camera (Diagnostic Instruments Inc., MI).

2.2.4 Image Processing

Region of Interest Segmentation and preprocessing

A 3D mouse atlas (Aggarwal et al., 2009) was used as a reference for segmenting anatomical regions-of-interest (ROI) using the segmentation editor in Amira® (FEI Software, OR). ROI included left/right: hippocampus (Hi), neocortex (Neo), olfactory bulb (OB), thalamus (Th), striatum (Str), hypothalamus (Hy), and brainstem (Stem).

The Analysis of Functional NeuroImages software (AFNI; http://afni.nimh.nih.gov/afni/) was utilized for all image processing (Cox, 1996). Resting-state fMRI data was co-registered to the anatomical MRI data using a two-step method in which an initial alignment using linear interpolation to smoothed variants of the
functional and anatomical datasets was followed by a final alignment step utilizing Fourier interpolation. The rsfMRI BOLD time course was then band-pass filtered from 0.01-0.08 Hz and spatially smoothed using a Gaussian filter with a full width half maximum (FWHM) of 0.5 mm. Data from the normal and tumor-bearing brains underwent identical image processing steps.

**Computation of Resting-State Connectivity**

For this data the effective TR=6.4 s, which results in a maximum sampling signal bandwidth of 0.078 Hz as dictated by the Nyquist-Shannon sampling theorem. We computed the average BOLD time course within each ROI for use as the ‘seed’ or ‘reference’ time course. The resting-state connectivity between any two ROI pairs was defined in terms of the cross-correlation between their respective average BOLD time courses.

**Visualization of Tumor-Induced Changes in Resting-State Connectivity**

Following cross-correlation analysis of the average resting-state BOLD time course for a given ROI with all the voxels for each segmented ROI, we created ‘resting-state’ maps for each brain. These maps are a visual representation of the connectivity between an ROI and the rest of the murine brain. Each resting-state map was thresholded at $p<0.05$ and overlaid on the anatomical image for visualization.

To make it easier to assess brain-tumor induced alterations in the resting-state ‘connectome’, we constructed radial plots and cross-correlation matrices. The radial
plot displays the resting-state connectivity between a single region of interest relative to the remaining segmented regions. Here, we computed a radial plot for the connectivity between the right hippocampus and other ROI for a representative healthy and tumor-bearing animal. The cross-correlation matrix displays the resting-state connectivity for each ROI relative to the rest of the murine brain. Row $i$ and column $j$ of the matrix is the resting-state connectivity between ROI pairs $i$ and $j$. Presented is the non-weighted average cross-correlation matrix for the healthy ($n=8$) and brain-tumor bearing ($n=8$) groups.

We also visualized the resting-state connectivity between murine brain regions as a force-directed spatial graph plotted using the Kamada-Kawai (KK) algorithm (Kamada and Kawai, 1989). In this spatial graph, each brain ROI was represented by a node and the strength of the connectivity between ROI represented by the thickness of the edge. The Kamada-Kawai algorithm computes the force-directed spatial separation between pairs of nodes (i.e. ROI). Therefore, the final spatial graph indicates the equilibrium state (i.e. net force = 0). Since the non-convex nature of the optimization problem can result in the final equilibrium position landing in an undesirable local maximum, we implemented multiple trials of this algorithm with random restarts. The end result was a spatial graph for each normal and tumor bearing brain. Here, we present the average spatial graph for each group using its respective non-weighted average cross-correlation matrix.

*Estimation of Power Spectral Density*
Periodograms were utilized to visualize the power spectral density of BOLD signal fluctuations in both healthy and tumor-bearing groups. The periodograms were obtained by calculating the squared-magnitude of the discrete Fourier transform (Oppenheim et al., 1999).

Statistical Analyses

To determine if there were significant differences in resting state connectivity between ROI pairs from healthy and tumor-bearing brains we employed a two-tailed, non-parametric Mann-Whitney U test ($\alpha = 0.05$). To determine if the resting state connectivity between ROI pairs varied as a function of tumor volume (i.e. tumor stage) we computed the non-parametric Spearman’s correlation coefficient ($\alpha = 0.10$).

2.3 Results

2.3.1 Visualization of brain tumor-induced alterations in resting state connectivity

Fig. 2.1a shows radial plots of the resting-state connectivity between the right hippocampus and ROIs of the segmented murine brain in a representative healthy (gray trace) and medium sized tumor-bearing brain (red trace). The radial plot for the healthy brain shows positive correlations between the right hippocampus and regions within the ipsilateral hemisphere, while negative correlations were observed for regions occupying the contralateral hemisphere. In contrast, the radial plot corresponding to the same ROI pairs for the brain tumor-bearing brain exhibited little or no correlation. Fig. 2.1c and 2.1e show anatomical images overlaid with the ‘resting-state’ map for the right
hippocampus, thresholded at $p < 0.05$, for a healthy and tumor-bearing brain, respectively. Figs. 2.1b and 2.1d are the segmented label fields for the ROI displayed in Figs. 2.1c and 2.1e, respectively. In the healthy brain, there was an inverse correlation between the right (white arrow) and left (orange arrow) hippocampus as can be seen in the map of Fig. 2.1c. In contrast, the presence of a brain tumor (T) attenuated the connectivity in the right hippocampus, i.e. the fraction of voxels exhibiting an inverse correlation between the left and right hippocampus decreased from 85.4% to 9.9% (Fig. 2.1e).

Figure 2.1: Visualization of alterations in resting-state connectivity induced by a medium sized brain tumor in the striatum. (a) Radar plot showing changes in resting-state connectivity between the right hippocampus and other ROIs for a normal (green) and tumor-bearing (red) brain. (b) Healthy brain label field overlaid over an anatomical slice, showing left hippocampus (orange arrow), right hippocampus (white arrow), left neocortex (dark blue – left), right neocortex (green – right), and brainstem (green – bottom). (c) Right hippocampus activation map overlaid on an anatomic slice for a normal brain. (d) Tumor-bearing label field overlaid on an anatomical slice showing tumor (red with white “T”), left
hippocampus (orange arrow), right hippocampus (white arrow), left neocortex (dark blue - left), and right neocortex (green). (e) Right neocortex activation map overlaid on an anatomical slice for a tumor-bearing brain. Tumor location is indicated by white hatched outline in (e). Head orientation (L=left, R=right, D=dorsal, V=ventral) indicated in the upper left corner of panels (b) and (d). Activation map overlays were thresholded at p < 0.05.

### 2.3.2 Brain tumors disrupt inter- and intra-hemispheric resting state connectivity

Overall, we observed that brain tumors induced a brain-wide reorganization of the resting state network that extended to the hemisphere contralateral to the tumor. Figs. 2.2a-b show the cross-correlation (CC) matrices averaged across healthy (n=8), and tumor-bearing (n=8) brains, respectively. The upper left quadrant of each matrix represents left intra-hemispherical connections; the lower right quadrant represents right intra-hemispherical connections; and the lower left quadrant represents inter-hemispherical connections. The CC matrix for the healthy brain (Fig. 2.2a) shows positive correlations between intra-hemispherical ROI pairs, for both the right and left hemispheres. However, inter-hemispherical connections were observed to be anti-correlated for many ROI pairs. In contrast, Fig 2.2b. shows that the patterns in resting-state connectivity found in Fig 2a. are not maintained in tumor-bearing brains. Specifically, the majority of pairwise connections tended to be random (i.e. 0), with some exceptions. Fig. 2.2c shows the ‘difference’ CC matrix that was generated by calculating the difference between the matrices shown in Fig. 2.2b and 2.2a. From this,
one can visualize the brain-wide alterations in resting-state connectivity as induced by brain-tumor.

Fig. 2.2d-e show the Kamada-Kawai plots corresponding to the CC matrices in Figs. 2.1a-b, respectively. The plot in Fig. 2.2d visually illustrates the correlation pattern observed in Fig. 2.2a. For example, healthy right and left hemisphere ROI occupy their own unique regions of the Kamada-Kawai space, implying out of phase connectivity. The thicknesses of the Kamada-Kawai plot edges in each sub region reflect the strength of the CC between ROIs inhabiting that space. In contrast, for the tumor bearing brains one can clearly see the loss of this spatial organization in the Kamada-Kawai plot shown in Fig. 2.2e. More specifically, one observes clustering of ROI and an overall lack of well-defined boundaries between hemispheres for the tumor-bearing brains. The global effect of brain tumor on the resting-state ‘connectome’ becomes more apparent when the corresponding Kamada-Kawai plots are overlaid as shown in Fig. 2.2f.

Fig. 2.3 summarizes the ROI pairs that exhibited significant ($p < 0.05$) tumor-induced alterations in resting-state connectivity. Here, Figs. 2.3a and 2.3b represent significantly affected ROI pairs lying within the left hemisphere and right hemisphere, respectively. Additionally, Fig. 2.3c represents significantly affected region pairs lying in opposing hemispheres. Connectivity was least likely to be altered in the left hemisphere (Fig 2.3a) because there was no brain tumor present there. Additionally, the majority of significant reorganization occurred in ROI occupying opposing hemispheres (Fig 2.3c).
Figure 2.2: Brain tumors disrupt inter- and intra-hemispheric resting state functional connectivity.

Correlation coefficient (CC) matrices illustrating the resting state functional connectivity for: (a) ROIs from healthy mice; (b) ROIs from brain tumor-bearing mice. (c) The ‘difference’ matrix between tumor-bearing and normal mouse ROIs illustrating the inter-ROI connectivity most affected by the presence of a tumor. Kamada-Kawai (KK) plots corresponding to the CC matrices in (a) and (b). (d) Average KK plot for normal mouse brains, (e) average KK plot for tumor-bearing mouse brains, and (f) an illustration of the alterations in connectivity between tumor-bearing and normal brains by overlaying the KK-plots in (d) and (e). Labels have been omitted in (f) for clarity. For each KK-plot, the edge thickness represents the strength of the connectivity between the nodes (i.e. ROI).
Figure 2.3: Box and whisker plots of the correlation coefficients between ROI pairs computed for normal and tumor-bearing brains. (a) ROI pairs in left hemisphere exhibiting significant differences; (c) ROI pairs in the right hemisphere exhibiting significant differences, and (c) ROI pairs in contralateral hemispheres exhibiting significant differences (n=8 for normal, n=8 for tumor p < 0.05).

2.3.3 Brain tumor progression (i.e. increasing tumor volume) alters resting-state connectivity

Changes in resting-state connectivity with brain tumor progression (i.e. increase in tumor volume) are shown in Fig. 2.4a-b for representative ROI pairs. Correlation between the left hypothalamus and right hypothalamus (Fig. 2.4a) decreased with tumor progression, while that between the left hippocampus and left striatum (Fig. 2.4b) increased. Fig. 2.4d and 2.4f show the connectivity maps for the right hypothalamus (hatched line) overlaid on a coronal anatomical slice of a brain bearing a small (2.67 mm$^3$) and large tumor (96.93 mm$^3$), respectively. Fig. 2.4c and 2.4e are the ROI label fields corresponding to Fig. 2.4d and 2.4f, respectively. The right hypothalamus (hatched line) resting-state map (Fig 2.4d) for the small tumor-bearing
brain shows significant positive correlations in the left hypothalamus; however, this was not the case in the right hypothalamus (hatched line) resting-state map for the large tumor-bearing brain (Fig 2.4f). Therefore, the presented resting-state maps reflect the trend of decreasing connectivity with increasing tumor volume as summarized in Fig. 2.4a. Tumor volumes are summarized in Fig 2.4g.

**Figure 2.4: Changes in resting state connectivity as a function of brain tumor progression.** (a) Nonlinear regression of the cross-correlation coefficient vs. tumor volume for the LHy-RHy ROI pair (n=8, p = 0.002). (b) Nonlinear regression of the cross-correlation coefficient vs. tumor volume for the LHi-LStr ROI pair (n=8, p=0.086). (c) Label field for small tumor-bearing subject overlaid on a coronal anatomical slice showing left neocortex (purple), right neocortex (light blue), left striatum (green), right striatum (yellow), left and right thalami (white), left hypothalamus (dark blue), and right hypothalamus (red). (d) Accompanying activation map of left hypothalamus overlaid on a coronal anatomical slice. (e) Label field for large tumor-
bearing subject overlaid on coronal anatomical slice showing left neocortex (purple), right neocortex (light blue), left striatum (green), right striatum (yellow), left and right thalami (right), left hypothalamus (dark blue), and right hypothalamus (red). (f) Accompanying activation map of left hypothalamus overlaid on the coronal anatomical slice. (g) Table of tumor volumes in ascending order. Activation maps were thresholded at p < 0.05. Orange arrows point to right hypothalamus, white arrows point to left hypothalamus. Hatched outline in activation maps (d),(f) represent right hypothalamus. Head orientation (L=left, R=right, D=dorsal, V=ventral) indicated in the upper left corner of panels (c) and (e). Tumor is located towards the posterior brain for both animals and therefore not visible in the presented slices.

2.3.4 Brain tumors attenuate the resting state BOLD signal across brain regions

Figs. 2.5 shows periodograms of the resting state BOLD signal for various ROI pairs in the right (Fig. 5a-f) and left hemisphere (Fig. 5g-l) averaged over both groups of animals. The average power of the resting state BOLD signal in the healthy brain was higher than that for tumor-bearing brains for all ROIs. Collectively, these data suggest that the variance in the resting-state BOLD signal was attenuated for the brain tumor group relative to the healthy group for all ROI.
Figure 2.5: Tumors suppress BOLD fluctuations across brain regions.

*Blood Oxygenation Level Dependent (BOLD)* periodograms dissect total BOLD signal variance into respective frequency contributions. Average BOLD time signal for each ROI was averaged across all healthy and tumor-bearing subjects. (a) BOLD periodograms in frequency range 0.01-0.08 Hz across all 13 ROIs for averaged healthy subjects (gray) and tumor-bearing subjects (red).

2.3.5 Converse correlation analysis and generation of “tumor connectivity” maps

Fig 2.6-b shows the “tumor connectivity” map that results by selecting the average resting-state BOLD time-course from a small tumor (2.67 mm³, thresholded at p
< 0.05) as a reference waveform for the cross-correlation analysis. Accompanying label fields are shown in Fig 2.6a, with tumor labeled in orange. Here, the entire tumor and adjacent cortical areas are significantly “connected” with each other.

Fig 2.6d shows the tumor connectivity map for a large tumor (122.89 mm³, thresholded at p < 0.05) with the accompanying label fields shown in Fig 2.6c, with tumor region highlighted in purple. One can clearly observe the tumor mass engulfing the majority of the right hemisphere. In contrast to the small tumor (Fig 2.6b), the tumor connectivity map for the larger tumor is mainly restricted to the periphery. More specifically, there are large portions of the tumor body that do not exhibit significant correlations. Furthermore, the contralateral hemisphere exhibits a degree of significant correlations when compared to contralateral hemisphere of the brain bearing the small tumor (Fig 2.6b).

Figure 2.6: Reverse analysis of “tumor connectivity” shows that the small tumor is synchronized with intrahemispheric ROIs and larger tumor synchronized with interhemispheric ROIs.
Label fields of small-tumor bearing subject. (b) Tumor resting-state connectivity map overlaid on anatomical slices. (c) Label fields of large-tumor bearing subject. (d) Tumor resting-state connectivity map overlaid on anatomical slices. Tumor in label fields is orange in (a) and purple in (c). Tumor connectivity maps in (b), (d) were thresholded at p < 0.05.

2.3.6 Brain tumors disrupt the neurovascular unit

Immunofluorescence labeling of key elements of the neurovascular unit allowed us to assess how brain tumor cells can remodel the neurovascular unit. To label functional tumor blood vessels, we intravenously administered dextran-TRITC (red channel), and labeled vascular basement membrane with anti-laminin (green channel). Fig 2.7a illustrates a region from a healthy brain displaying intact neurovascular basement membrane coverage in intimate contact with dextran-TRITC perfused blood vessels. In contrast, brain-tumor region presented in Fig 2.7b shows some perfused tumor blood vessels that are devoid of laminin staining, indicating that the tumor vasculature had abnormal or incomplete basement membrane.

Additionally, astrocytes and pericytes were stained using anti-GFAP and anti-α-smooth muscle actin antibodies, while intravenously administered dextran-TRITC was used to label functional blood vessels. Fig 2.7c and 2.7d compare the hippocampal region from a healthy and tumor-bearing mouse brain, respectively. In Fig 2.7c, we clearly observed overlap of astrocytes (green channel) with dextran-TRITC perfused vessels (red channel), indicative of robust astrocytic-vascular coupling. However, Fig 2.7d shows that this was not the case for the tumor region (T) as indicated by the lack of astrocytes (green channel). Moreover, it should be noted that astrocytic-vascular
coupling appeared to be physically intact outside of tumor region. **Fig 2.7e** provides a magnified view of the tumor (T)-cortical interface (hatched line) wherein one can juxtapose the stark difference in astrocytic coverage of the neurovasculature between the tumor and non-tumor regions. Specifically, there is intimate contact between astrocytic end-feet (green channel) and blood vessel endothelium (red channel) on the cortical side of the interface, in contrast to an absence of astrocytic coverage on the tumor side. Additionally, **Figs 2.7f** shows intimate astrocytic coverage (green channel) of blood vessels (red channel) in healthy brain, while a magnified view of the tumor region in **Fig 2.7g** shows an absence of astrocytic coverage. Finally, **Fig 2.7h** illustrates that analogous to the stark differences in astrocytic coverage between the healthy and tumor brain regions, there were also differences in smooth-muscle coverage. The tumor vasculature exhibited tenuous smooth muscle coverage or the smooth muscle was dissociated from the vasculature. In contrast, there was intimate smooth muscle coverage of the cortical vasculature. Collectively, these histologic data indicate brain tumor-induced remodeling of the neurovascular unit.
Figure 2.7: The neurovascular unit is disrupted in brain tumors. Optical microscopy images of tissue sections from healthy and brain tumor-bearing murine brains in which elements of the neurovascular unit have been labeled immunofluorescently. (a) Healthy brain region in which neurovascular basement membrane has been labeled with laminin (green channel) and perfused blood vessels with i.v. administered dextran-TRITC (red channel). Once can see that all the blood vessels exhibit complete laminin coverage indicative of an intact basement membrane. In contrast, (b) brain tumor bearing regions have blood vessels (red channel) that do not exhibit laminin staining (green channel) and is indicative of tumor vasculature that has an incomplete or abnormal basement membrane. (c) Hippocampal region from a healthy mouse brain in which astrocytes have been labeled with GFAP (green channel) and perfused blood vessels with dextran-TRITC (red channel). Tumor (T) and surrounding region in which astrocytes have been labeled with GFAP (green channel) and blood vessels with dextran-TRITC (red channel). One can observe an almost complete absence of GFAP labeling within the tumor region. High magnification fields of: (e) the brain tumor (T)-cortical interface (hatched line) region wherein one can observe intimate contact between the astrocytes and blood vessels on the cortical side of the interface and an absence of astrocyte coverage on the tumor side. (f) Intimate astrocyte coverage (green channel) of cortical blood vessels (red channel) in healthy murine brain, and (g) absence or incomplete astrocyte coverage of tumor blood vessels. (h) Tumor-cortical interface showing extensive pericyte (green channel) of the cortical blood vessels (red channel) and
incomplete or sparse pericyte coverage of tumor blood vessels. Collectively, these histological data demonstrate disruption of the neurovascular unit in brain tumor-bearing brains. Scale bar = 50 µm in all panels.

2.4 Discussion

Relating the underlying physiology to the observed contrast in MRI has been the subject of intensive research within the context of the tumor microenvironment (TME) (Emblem et al., 2013; Pathak et al., 2008). To date, a number of preclinical studies using tumor-xenograft models have sought to elucidate the effects of the TME on the BOLD fMRI signal. The works of Baudelet et al and Goncalves et al related spontaneous $T_2^*$ signal fluctuations with heterogeneity in tumor blood flow (Baudelet et al., 2006; Goncalves et al., 2015). More specifically, they found that tumor vessel functionality, i.e. limited perfusion, was directly responsible for the cyclic-hypoxia experienced throughout the tumor. Additionally, analysis using ICA-decomposition (Goncalves et al., 2015) enabled Goncalves et al to separate tumor-specific from systemic (or global) $T_2^*$ fluctuations. While, these findings have provided a useful benchmark for understanding the role of the TME on baseline BOLD signal fluctuations, to the best of our knowledge this is the first time an orthotopic (i.e. occurring at the normal place in the body) brain tumor model (Vakoc et al., 2009) has been employed to better understand the role of the ‘native’ angiogenic brain tumor TME on the resting-state BOLD signal.

Much of our current knowledge of the interaction between brain tumors and the BOLD fMRI signal comes from clinical studies. Holodny et al were one of the first groups
to recognize that reduced task-based functional activation could be the result of dysfunctional vascular autoregulation in brain-tumors (Holodny et al., 1999). Specifically, they observed reduced task-based activation of the motor cortex in the hemisphere containing the glioblastoma tumors. Ulmer et al also reported reduced BOLD activation near and surrounding glial tumors in response to a language-task, and suggested the role of neurovascular uncoupling in their observations (Ulmer et al., 2003). Additionally, recent evidence suggests that lack of vascular autoregulation resulting from brain-tumor induced neurovascular uncoupling can disrupt resting-state connectivity between brain regions, and result in false-negatives on resting-state maps. For example, Agarwal et al characterized changes in resting-state connectivity in a clinical study with seven patients with an array of brain-tumors including glioma, glioblastoma, and oligoastrocytoma (Agarwal et al., 2016). Regions of the brain encompassing bilateral motor and sensorimotor networks exhibited reduced connectivity in the brain tumor-bearing hemisphere relative to the healthy contralateral hemisphere.

Lack of vascular autoregulation may also explain other clinical studies that have sought to characterize the BOLD signal profile of brain tumors relative to non-tumor-bearing brain regions. For example, studies on brain tumors such as glioblastomas and meningiomas have demonstrated that tumors exhibit a markedly different resting-state BOLD signal when compared to surrounding tissue (Chow et al., 2016; Feldman et al., 2009). Chow et. al also showed that in glioblastoma the temporal profile of the BOLD signal exhibited some overlap with that from peritumoral regions (Chow et al., 2016).
Collectively, these findings suggest that while tumors disrupt resting-state connectivity, they can also exhibit their own unique and intrinsic BOLD temporal profile.

The global effects of brain tumors on resting-state brain connectivity has been investigated only recently (Maesawa et al., 2015). These authors not only discovered loss of connectivity in default mode and executive control networks, but also correlated their observations with reduced cognitive function in patients. Preclinical studies such as ours can expand on such novel findings, by helping to correlate macroscopic observations of tumor induced reorganization of the resting-state connectome with changes in the underlying TME. Currently, it is unknown if brain tumor progression coincides with neurovascular uncoupling. Nor is it known how this uncoupling modulates the BOLD rsfMRI signal, or if this uncoupling varies as a function of tumor grade and vascular phenotype. Since it is challenging to obtain such data from patients, in this preclinical study we resorted to the well-characterized orthotopic 9L brain tumor (Barth and Kaur, 2009) model to elucidate how resting-state BOLD signal fluctuations are locally and globally modulated by the presence of a brain tumor.

We observed that brain tumors disrupt the global resting-state ‘connectome’. Collectively, our resting-state connectome data derived from healthy murine brains matched that observed in healthy mice from multiple other rsfMRI studies (Jonckers et al., 2011; Mechling et al., 2014). Using ICA analysis, these groups demonstrated that murine brains do not exhibit bilateral symmetry in many resting-state networks, unlike their rat counterparts (Jonckers et al., 2011). It should be pointed out that recent
resting-state studies using optical intrinsic signal (OIS) and laser speckle contrast (LSC) imaging in mice have demonstrated bilateral resting-state connectivity (Nasiriavanaki et al., 2014; Nasrallah et al., 2014; White et al., 2011). This difference might be attributable to the limited cortical depth of coverage (< 1 mm) of these widefield optical imaging approaches relative to the whole-brain coverage obtainable with in vivo MRI. Moreover, differences in the in-plane spatial resolution, ranging from microns for optical methods to sub-millimeter for rsfMRI techniques make it challenging to directly juxtapose these results. Currently, we are also in the process of mapping the resting-state connectome of the murine brain at the microvascular-scale using LSC and OIS to determine how it is affected by spatiotemporal smoothing, imaging resolution, and brain tumor progression (Senarathna et al., 2017).

The concept of “small-world” networks, as reviewed by Sporns and Honey, also supports our current observations of hemispheric lateralization in the brains of healthy mice (Sporns and Honey, 2006). This idea of a small-world topology postulates that the complex networks of the brain are characterized by dense local clustering between closely neighboring functional regions, whereas few long-range connections exist (Bassett and Bullmore, 2006). Experimental evidence of this has been revealed via rsfMRI. Specifically, Salvador et al uncovered dense local connections between visual, somatosensory, and auditory-verbal clusters, whereas sparse connections were found between ventral and dorsal visual regions (Salvador et al., 2005a; Salvador et al., 2005b). Although a recent review has recommended “thresholding” away negatively correlated brain regions (Rubinov and Sporns, 2010), we did not adopt this approach for the
current study. The results mentioned above, in conjunction with our observation that the majority of affected pairwise connections occurred between regions occupying opposing hemispheres, suggests a possible role of certain brain regions in “channeling” resting-state activation between hemispheres. If so, such a conduit is clearly disrupted by brain-tumor progression. It should be noted that this observation was only possible because we did not threshold negatively correlated brain regions, allowing us to visualize the murine connectome in its entirety. With finer brain ROI segmentation, researchers will be afforded the opportunity to uncover the effects of brain-tumor induced neurovascular uncoupling over a wider range of brain regions than the ones used here. In addition, network analysis tools (Rubinov and Sporns, 2010) on force-directed spatial graphs produced from a larger number of segmented regions may allow us to refine our understanding of this disease in terms of neuronal networks.

The neurovascular coupling mechanism, as first described by Roy and Sherrington, is crucial for neuronal populations to meet their energy demands prior to activation (Roy and Sherrington, 1890). Recent studies at the cellular scale have demonstrated that invading glioma cells are capable of disrupting this astrocyte-vascular coupling and the blood-brain barrier (Lee et al., 2009; Watkins et al., 2014). Additionally, task-based fMRI studies in conjunction with breath-hold based cerebrovascular reactivity (CVR) mapping in patients have demonstrated that neurovascular uncoupling can also occur in early stage brain tumors (Pillai and Zaca, 2011). These observations are consistent with our in vivo rsfMRI data as well as our histological data showing a dysfunctional neurovascular unit. This neurovascular uncoupling histologically manifests
itself in terms of abnormal vascular basement membrane, and sparse or absent astrocytic and pericyte coverage. Our comparison of the power of the BOLD rsfMRI signal fluctuations between healthy and tumor-bearing groups highlighted a brain-wide and significant attenuation of the BOLD signal in the latter. Collectively, the in vivo rsfMRI and histologic data indicate that an inability to autoregulate vasodilation at a global level may be responsible for the observed tumor-induced reorganization of the resting-state ‘connectome’.

We also investigated the effects of tumor progression on resting-state connectivity between brain regions. We presented two cases in which significant changes in resting-state connectivity were modeled as a function of tumor volume. The connectivity between the left and right hypothalamus decreased with tumor progression, while that between the left hippocampus and left striatum increased with tumor volume. A possible explanation for this is that the mass effect of large tumors can often result in edema, which has been known to occur along white matter tracts, and disrupt the connectivity between brain regions (Weissman, 1988). Another possibility is that the mass effect of the progressing tumor and its accompanying angiogenic vascular bed as it breaches the interhemispheric boundary either via the ventricles or the corpus callosum can induce new regions of connectivity. This can potentially occur via the mechanisms of vascular co-option and/or invasion by brain tumor cells (Lee et al., 2009). These observations indicate that the relationship between tumor progression and modulation of the resting-state connectome depends on the location and spatial extent
of the tumor. This is worth bearing in mind when contemplating the use of rsfMRI as a potential cancer biomarker (O’Connor et al., 2016).

In this study, we also conducted the converse analysis to map “tumor connectivity” using a reference time course averaged over the entire tumor volume. We observed that in general, large and small brain tumors exhibited unique rsfMRI signal profiles relative to non-tumor ROI. These observations are consistent with those from recent clinical studies exploring the relationship between tumor and non-tumor BOLD signals (Chow et al., 2016; Feldman et al., 2009). We observed that the majority of small tumor voxels displayed high correlation with the average tumor BOLD signal; this was not the case for the large tumor. One reason for this may be poor oxygen delivery or hypoxia in larger tumors, wherein it has been suggested that pO2 concentrations are highest in the periphery or well-vascularized angiogenic rim of the tumor (Hallac et al., 2014). Additionally, recent research has demonstrated that brain tumors of varying sizes may show differences in their underlying mechanisms of neurovascular uncoupling. Specifically, it has been suggested that neurovascular uncoupling in smaller tumors may be the result of astrocytic dysfunction (Zaca et al., 2014), whereas that in large tumors may be primarily the result of tumor angiogenesis and dysfunctional new tumor vasculature (Jiang et al., 2010). Dysfunctional tumor vasculature has already been implicated in cyclic hypoxia and baseline BOLD fluctuations in vivo (Baudelet et al., 2006; Goncalves et al., 2015). This same phenomenon might explain the sparsity of resting-state synchrony in the large tumor. Lastly, ICA analysis has shown that systemic-specific $T_2^*$ fluctuations were directly correlated with tumor volume (Goncalves et al., 2015).
While it is difficult to compare two different tumor models, the observed synchronization differences of the BOLD rsfMRI signal in small and large-tumors relative to the contralateral brain are in line with observations reported by Goncalves et al (Goncalves et al., 2015).

While the use of isoflurane anesthesia was necessary for reducing head motion, we are aware of the possible effects of volatile anesthetics on the computation of resting-state maps. Recent studies in mice have suggested that the use of isoflurane as an anesthetic may be preferable to α-chloralose or medetomidine for fMRI studies because the latter significantly impacts physiological factors such as heart rate and blood pH (Low et al., 2016). It has also been shown that administration of isoflurane does not impact rsfMRI connectivity between brain regions (Grandjean et al., 2014; Jonckers et al., 2014). Moreover, Guilfoyle et al demonstrated that although all anesthetic agents may alter cerebral blood flow and cerebral metabolism, reliable functional connectivity measures in mouse brain can still be obtained with isoflurane (Guilfoyle et al., 2013). However, we are aware that inhalation of isoflurane can induce correlations between the olfactory bulb and neocortex. In addition, the variance we observed in the resting-state connectivity of brains of healthy mice could be due to small inaccuracies in ROI delineation resulting from manual segmentation of the murine brain. While one could use automated registration and segmentation tools such as the large deformation diffeomorphism metric mapping algorithm (Christensen et al., 1996), the presence of a brain tumor in the murine cortex precludes such an approach in the tumor-bearing brains. This issue is compounded by small variations in tumor location.
and large variations in tumor volume. Therefore, we resorted to manual segmentation of the murine brains by two independent operators.

In summary, we have demonstrated the global effects of brain-tumor volume on resting-state BOLD fluctuations and resting-state connectivity between brain regions in a preclinical model. By comparing the brains of healthy and tumor-bearing mice, we discovered that tumor-induced neurovascular uncoupling attenuates resting-state BOLD signal fluctuations and results in reorganization of the resting-state ‘connectome’. These results may help explain the loss of cognitive function in brain tumor patients in areas extending beyond the tumor region. We expect the refinement of this work with preclinical models of different brain tumor phenotypes will facilitate the development of rsfMRI as a new clinical biomarker for brain cancer.
Given the findings in the previous chapter, we sought to utilize microvascular-resolution optical imaging to probe deeper into tumor induced neurovascular uncoupling and its effects on cerebrovascular dynamics. We present our newly developed analysis techniques and preliminary findings.

3.1 Introduction

To meet cerebral metabolic demand, a complex neurovascular mechanism is responsible for coupling neuronal activation with modulation of vascular tone and local cerebral blood flow (CBF) (Roy and Sherrington, 1890). Disruptions in this mechanism, termed neurovascular uncoupling (NVU), are significant for three reasons:

(i) NVU has been shown to accompany declining cognition induced by the aging process (Ungvari et al., 2017) and in brain diseases such as Alzheimer’s (Kisler et al., 2017);

(ii) impaired cognitive function was recently correlated with disruptions of resting state networks induced by brain tumor progression (Maesawa et al., 2015);

(iii) the hostile tumor microenvironment (TME) of brain tumors is often associated with perturbations of the neurovascular unit (Lee et al., 2009; Pak et al., 2017).

47
Overall, this has sparked widespread research into functional imaging approaches capable of early detection of brain tumor-induced alterations in brain functions.

Research has shown that the complex interplay between abnormal brain tumor vasculature (Kim et al., 2011), blood flow characteristics (Jain et al., 2007), and brain tumor-induced neurovascular uncoupling (NVU) (Pak et al., 2017) can confound the interpretation of functional imaging studies (Agarwal et al., 2016; Ulmer et al., 2003). Collectively, this leaves clinicians unable to determine if attenuation of the blood oxygen level dependent (BOLD) functional MRI (fMRI) signal is due to NVU or due to a lack of neuronal response resulting from brain tumor progression. To investigate this open question, we previously utilized the resting state fMRI (rsfMRI) signal, which is based on the premise that distinct brain regions exhibit temporally coherent spontaneous fluctuations in blood flow (Biswal et al., 1995) that often follows neuronal activation (Ma et al., 2016). Overall, we observed global changes in resting state connectivity as well as attenuation of the resting state BOLD signal over tumor progression. Histological evidence confirmed that the neurovascular unit was disrupted in tumor bearing brains.

However, the low spatial and temporal resolution inherent to MRI techniques left us unable to pinpoint the underlying mechanisms responsible for such changes. For example, while it is the arterioles which have been shown to respond to neuronal activation via neurovascular coupling (Hillman, 2014), the underlying biophysics of the BOLD signal (Ogawa et al., 1990) makes it such that venous compartments are responsible for the majority of BOLD signal variance (Roberts et al., 2007). Being able to probe individual vessels would be a useful step towards bridging local cerebrovascular
dynamics and global functional networks, and would lead to a more complete understanding of the relationship between brain tumor progression, NVU, and changes in neuronal function. To begin to tackle this, we proposed the use of microvascular resolution wide-field optical imaging.

Converging evidence has suggested that resting state connectivity mapping of the healthy murine neocortex using optical imaging yields results congruent with fMRI research (Bergonzi et al., 2015; Jonckers et al., 2011). Therefore, in this preclinical study we seek to utilize wide-field optical imaging of multiple hemodynamic parameters such as cerebral blood flow (CBF) acquired through laser speckle contrast imaging (LSCI) (Senarathna et al., 2013) and cerebral blood volume (CBV) acquired through optical intrinsic signal (OIS) imaging (Hillman, 2007) to further characterize brain tumor induced disruptions in cerebrovascular dynamics. Here, we showcase preliminary investigations on tumor induced alterations in resting state connectivity relative to healthy brains, while also characterizing the resting state temporal-frequency dynamics in tumor affected tissue. Additionally, we subjected mice to a carbogen gas (5% CO₂, 95% O₂) challenge (Cain et al., 2013) in order to gauge vascular functionality. In modeling the hemodynamic response function (HRF) to carbogen inhalation, we characterized differences in hemodynamics in tumor tissue relative to contralateral brain. Additionally, we suggest the use of an ‘entropy’ metric to isolate brain tissue affected by brain tumor. Lastly, we developed a pipeline to separate arterioles from venules by modeling tracer kinetics from dextran-TRITC tail vein injection (Thompson et al., 1964), allowing us to probe tumor induced alterations at the level of individual arterioles and
venules. Collectively, this chapter provides a platform for a future peer-reviewed manuscript on this topic.

3.2 Methods

3.2.1 Animal Preparation

9L-GFP brain tumor cells were orthotopically inoculated into the cortices of SCID mice \( n=5 \). Brains of healthy SCID mice \( n=5 \) served as the control group. Briefly, severe combined immune deficient (SCID) mice weighing 25 – 30g (Charles River/NCE, Frederick, MD), were anesthetized with a xylazine/ketamine cocktail for tumor cell inoculation. Their heads were immobilized and using an aseptic technique, a 1mm burr whole drilled in the skull between 1.5 mm anterior of the bregma and 1.5 mm left of the sagittal suture. A 10 \( \mu \)L gas-tight syringe (Hamilton Comp, Reno, Nevada) was used to inject 200,000 cells of 9L-GFP gliosarcoma cells 2mm deep into the brain. The 9L cell line was obtained from the Brain Tumor Biology Laboratory, and grown in DMEM (Gibco, Gaithesburg, MD) with 10% FBS. Cells were expanded immediately prior to inoculation. The injection time was 5 min, after which the needle was retracted slowly for an additional 5 min. The skin was then closed with surgical staples that were removed prior to optical imaging. All animal studies were performed according to institutional guidelines and the NIH “Guide for the Use and Care of Laboratory Animals”.

3.2.2 In Vivo Imaging protocol with Intact Skull
In preparation for imaging, mice were anaesthetized with isoflurane (2-2.5% mixed with air and oxygen, 1L/min flow rate). The mice’s eyes were covered with ointment and fur removed from the skull. Next, the skin was sterilized and then cut to expose the skull. Connective tissue was then removed. Once the skull dried, cyanoacrylate was applied to the perimeter and the mouse was fitted with a 3D custom printed headmount. After the cyanoacrylate had dried, dental cement was applied around the inside edge of the well in order to reduce water leakage, and the well was filled with saline to keep the skull moist. A glass cover slip was placed on top of the well to seal it off.

All animals with and without brain tumor were imaged in vivo on a multi-contrast benchtop system (Fig 3.1a) under isoflurane anesthesia (2-2.5% mixed with air and oxygen, 1L/min flow rate). Tumor bearing animals were imaged 7-10 days after inoculation. We utilized a white light source and filter wheel to acquire 570nm OIS images for mapping cerebral blood volume (CBV), a 632nm red laser to perform LSCI for mapping cerebral blood flow (CBF), and a 532nm green laser to image dextran-TRITC tracer kinetics. Images were captured via a 16-bit charged coupling device (CCD) camera and connected to a master data acquisition program in MATLAB (www.mathworks.com). The temporal resolution of the system was approximately 10 frames per second (fps). The spatial resolution was 6 µm x 6 µm, and high enough to resolve the cerebrovasculature. The ‘wide’ field of view (FoV) of 8.4 mm x 6.6 mm enabled acquisition of the entire murine neocortex.
The imaging protocols were as follows: (i) Each mouse underwent two 15-20 minute resting state imaging sessions using 570nm OIS imaging and LSCL. (ii) To access vascular functionality, carbogen gas was delivered to the mouse via inhalation. Here, each mouse underwent two 30-minute imaging sessions to map cerebral blood flow (CBF) and cerebral blood volume (CBV) with the following experimental protocol: 10 minutes room air, 10 minutes carbogen inhalation (1 L/min flow rate), and 10 minutes room air. (iii) Lastly, each mouse was perfused with the intravascular tracer dextran-TRITC (155 kDa, Sigma Aldrich, MA) via tail vein injection, with simultaneous green laser imaging, in order to distinguish between arteries and veins. Example whole-brain maps of CBV and CBF are shown in Fig 3.1b.

3.2.3 Assessing brain tumor growth

After in vivo imaging, animals were euthanized, brains excised and fixed in 10% buffered formalin solution. To isolate brain tumor position and to access if growth had occurred, we imaged the excised brain on a Nikon ECLIPSE-TS100 microscope (Nikon Instruments Inc., NY) with blue light excitation for detecting GFP signal (green channel). Lack of GFP signal indicated that the tumor may be too deep for light to penetrate. To resolve this, we utilized the ScaleS (Hama et al., 2015) protocol for whole brain clearing. First, permeability of the sample was enhanced by incubation in ScaleS0 solution containing 20% sorbitol, 5% glycerol, 1 mM methyl-beta-cyclodextrin, 1 mM gamma-cyclodextrin, 1% N-acetyl-L-hydroproline, and 3% DMSO for 12 hours. Methl-beta-cyclodextrin and gamma-cyclodextrin extract cholesterol from biological membranes.
whereas N-acetyl-L-hydroxyproline loosens collagen structures. It was noted that incubation in ScaleS0 rendered the fixed sample amenable to tissue clearing solutions. This adaptation process was achieved in the original ScaleA2 protocol by a freeze/thaw procedure that involved cryoprotection, OCT embedding, and re-fixation. These time-consuming and laborious steps in the original protocol were replaced by a simple incubation in ScaleS0. Secondly, the permeable (adapted) sample was incubated sequentially in ScaleS1, ScaleS2, and ScaleS3. These urea-containing and salt-free ScaleS solutions gradually clear the sample. Finally, the sample is restored by simple washing with PBS(-) (descaling) for at least 6 hours, and then incubated (37 degrees C) in ScaleS4 for 12 hours prior to observation. Fresh ScaleS4 is used as the mounting medium. The experimental timeline can be found in Table 3.1. After clearing, adjacent 1 mm brain sections were cut, placed on silanized slides, and re-imaged.

<table>
<thead>
<tr>
<th>Action</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation</td>
<td>4% PFA/PBS(-) (4°C)</td>
</tr>
<tr>
<td>Adaptation</td>
<td>ScaleS0 (~12 hrs, 37°C)</td>
</tr>
<tr>
<td>Permeabilization</td>
<td>ScaleS1 (~12 hrs, 37°C)</td>
</tr>
<tr>
<td></td>
<td>ScaleS2 (~12 hrs, 37°C)</td>
</tr>
<tr>
<td></td>
<td>ScaleS3 (~12 hrs, 37°C)</td>
</tr>
<tr>
<td>DeScaling</td>
<td>PBS(-) (~12 hrs, 4°C)</td>
</tr>
<tr>
<td>Clearing</td>
<td>ScaleS4 (~12 hrs, 37°C)</td>
</tr>
<tr>
<td>Mounting</td>
<td>ScaleS4 (RT)</td>
</tr>
</tbody>
</table>

*Table 3.1: ScaleS experimental timeline*
3.2.4 Image preprocessing

Laser speckle contrast

In order to calculate the speckle contrast ($k$), a 3x3 neighborhood was used to determine the mean light intensity and standard deviation at each pixel $(x,y)$. Speckle contrast was then calculated as (Senarathna et al., 2013):

$$k(x, y) = \frac{\sigma(x,y)}{\mu(x,y)}$$  \hspace{1cm} (1)

Here, $\mu$ and $\sigma$ are the mean and standard deviation of light intensity at each pixel centered in the 3x3 window. The speckle contrast ($k$) is related to blood flow speed by (Senarathna et al., 2013):

$$k^2 = \frac{T}{\tau} \left\{ 2 - \frac{T}{\tau} \left[ 1 - \exp \left( -\frac{2T}{\tau} \right) \right] \right\}$$  \hspace{1cm} (2)

Here exposure time is denoted by $T$ and $\tau$ denotes the decorrelation coefficient, a quantity inversely proportional to blood flow speed. For small $\tau$ values (associated with typical microcirculation imaged by our microscope), Eq. 2 can be simplified to:

$$\frac{1}{\tau} \propto \frac{1}{k^2}$$  \hspace{1cm} (3)

Eq. 3 was used to compute relative CBF.

Resting state, carbogen, and Dextran-TRITC preprocessing steps

CBV and CBF maps acquired in both the resting state and carbogen protocols were down-sampled to 1 second temporal resolution. (i) The resting state time courses
were bandpass filtered from 0.02 – 0.08 Hz (MATLAB). (ii) For the carbogen protocol, maps of CBV and CBF dynamics were converted to percent change from baseline (ImageJ):

$$r(t) = \frac{x(t) - \bar{x}_B}{\bar{x}_B}$$  \hspace{1cm} (4)

Here, $r(t)$ is the calculated response measured as the percent change from baseline, $x(t)$ the measured variable, and $\bar{x}_B$ is the baseline (0 – 10 s) mean of the measured variable. (iii) Lastly, for the dextran-TRITC tail vein injection protocol, the image stack was low-pass filtered at a cutoff of 1.5 Hz (MATLAB). Images acquired from all protocols were down sampled by a factor of 2 in each dimension, yielding an effective spatial resolution of 12 μm x 12 μm for CBV and dext-TRITC maps, and 36 μm x 36 μm for CBF maps.

3.2.5 Registration and Region of Interest Segmentation

We used a scale-rotation registration procedure (ImageJ) to register the CBF resting state, CBV carbogen, CBF carbogen, dex-TRITC kinetics, and tumor-GFP stacks to the mean resting state CBV image stack. First, transformations between the mean resting state CBV map and the mean of the image stacks mentioned above were found. For dex-TRITC tracer kinetics, we used the maximum intensity projection (MIP). Then, these transformations were applied to all images in the respective stacks.

Next, a 10 μm x 10 μm hierarchical 2D atlas of the mouse neocortex (http://mouse.brain-map.org/static/atlas) (Fig 3.2) was used as a reference for
segmenting anatomical regions-of-interest (ROI) using the segmentation editor in Amira® (FEI Software, OR). To register the atlas to the mean CBV map acquired from the resting state protocol, we utilized the Bookstein (Bookstein, 1989) thin plate spline based landmark (8-12 landmarks) registration (Amira). The ROIs identified included the left/right (L/R) primary motor (MotorP), L/R secondary motor (MotorS), L/R somatosensory (Somato), L/R auditory (Aud), L/R anterolateral visual (ALVis), L/R anteromedial visual (AMVis), L/R primary visual (VisP), L/R retrosplenial (Retro), anterior cingulate (AC), and prelimbic (PreL).

3.2.5 Data Analysis

Computation of Resting State Connectivity Maps

From here, all image stacks were imported into AFNI (http://afni.nimh.nih.gov/afni/) (Cox, 1996). To compute resting state connectivity maps, we first subjected cerebral blood flow (CBF) images to a spatial mean filter with sliding window of 30 x 30 pixels, and cerebral blood volume (CBV) images to a spatial mean filter with a sliding window of 90 x 90 pixels to create an effective spatial resolution of 1.08 mm x 1.08 mm for both image stacks. This resolution is comparable to that of fMRI techniques and is equivalent to current optical imaging studies of resting state connectivity (Bergonzi et al., 2015). This is the only stage in the analysis where hemodynamic maps underwent spatial filtering. Next, we regressed the global mean signal from each voxel. We then computed the average resting state CBF and CBV time courses within each ROI for use as the ‘seed’ or ‘reference’ time course. The resting-
state connectivity between any two ROI pairs was defined in terms of the cross-correlation coefficient between their respective average resting state time courses.

**Visualization of Tumor-Induced Changes in Resting-State Connectivity**

Resting state connectivity maps are a visual representation of the connectivity between an ROI and the rest of the murine brain. To create this, the average hemodynamic time course from a given ROI was cross-correlated with every pixel in the brain. Connectivity maps were thresholded at $p<0.001$ (Bonferroni corrected) and overlaid on the mean resting state CBV map.

Furthermore, we constructed cross-correlation matrices to display the resting state connectivity for each ROI relative to the rest of the murine brain. Row $i$ and column $j$ of the matrix is the resting-state connectivity between ROI pairs $i$ and $j$.

**Time-frequency analysis of resting state dynamics**

To visualize the temporal stability of resting state CBF and CBV dynamics in various regions of interest, spectrograms were utilized. Resting state CBF and CBV maps underwent a 3x3 temporal median filter to remove outliers. Next, spectrograms were acquired by computing periodograms, an estimate of power spectral density, and calculated by taking the squared-magnitude of the discrete Fourier transform (Oppenheim and Schafer, 1975), within a moving window with five-minute epochs and sequential five-second shifts. Each vertical stripe in the spectrogram is the calculated periodogram for a given five-minute epoch. The color map represents the estimated
power of the resting state signal at a given frequency (y-axis) and time (x-axis). To condense the information presented by the spectrogram, Welch’s method (Welch, 1967) was used to calculate the mean power and standard deviation at each discrete frequency element over all periodograms. Non-linear regression using *fitnlm* (MATLAB) with the following model (Eq. 5) was used to fit the mean periodogram calculated from Welch’s method. The 1/f distribution is intrinsic to the resting state frequency distribution (Cordes et al., 2001). Coefficient of determination ($R^2$) was calculated to assess goodness of fit.

\[
y = a_1 \frac{1}{x} + a_2
\]  

(Eq. 5)

Here, $y$ is power in arbitrary units, $x$ is frequency in hertz, and $a_1$ and $a_2$ are the best-fit parameters acquired from the fitting procedure.

**Modeling the HRF in response to carbogen inhalation**

In this analysis, we sought to compare and contrast the hemodynamic response function (HRF), which reflects the modulation of vascular diameter in response to carbogen intake, in tumor vs non-tumor tissue. We utilized a double gamma-variate waveform (Lindquist et al., 2009) to model, per-pixel, the HRF (Eq. 6) relating the hemodynamic and ‘box-car’ carbogen stimulus waveforms. The HRF function is parameterized by 7 variables – amplitude ($A$), time to initial response of first and second gamma-variates ($t_{o,1}$ and $t_{o,2}$, respectively), shape-parameters ($r_1$ and $r_2$), and scale-parameters ($b_1$ and $b_2$).
\[ H(A, t_{o,1}, r_1, t_{o,2}, r_2, b_1) = \begin{cases} A [ (t - t_{o,1})^r_1 e^{-\frac{(t-t_{o,1})}{b_1}} - \frac{i}{e} (t - t_{o,2})^{r_2} e^{-\frac{(t-t_{o,2})}{b_2}} ]; & t \geq t_{o,1} \\
0; & \text{otherwise} \end{cases} \]

To acquire the optimal HRF, an initial guess on the seven parameters was made and optimized via the `fminsearchbnd` function in MATLAB. We assumed that the amplitude parameter must be greater than zero. This optimization technique uses an iterative, derivative-free approach to minimize the sum-of-squares difference (i.e. maximize the fit) between the hemodynamic waveform and the stimulus box-car waveform convolved with the HRF transfer function (Eq. 7).

\[ C(f, H, g) = \sum_{i=1}^{T} (g_i - (H * f)_i)^2 \]

Here, \( C \) represents the cost-function that is to be minimized, \( g \) is the hemodynamic signal, \( f \) is the stimulus box-car waveform, \( H \) is the HRF, and ‘\( * \)’ is the convolution operator.

\( R^2 \) was calculated to access how well HRF convolved with carbogen box-car model fit the original hemodynamic waveforms.

**Calculating entropy**

Here, we sought to integrate the parameters of the HRF, acquired from the modeling approach above, into a single metric that may be useful for detecting superficial brain tissue affected by brain tumor. We noticed that the spatial map for parameters of the second gamma-variate function (Eq. 6) were heterogeneous.

Therefore, we calculated an ‘entropy’ metric from these parameters, presented in Eq. 9
and calculated in MATLAB. In this equation, $r$ and $b$ are the shape and scale-parameters, respectively, for the second gamma-variate function in Eq. 6.

$$E(r, b) = (r + 1) + ln(b) + ln\left(\Gamma(r + 1)\right) + (-r)\Psi(r + 1)$$ \hspace{0.5cm} (9)

Here, $E$ represents entropy of the gamma-variate function, $\Gamma$ is the gamma function (www.mathworks.com/help/matlab/ref/gamma.html), and $\Psi$ is the tri-gamma function (www.mathworks.com/help/matlab/ref/psi.html).

**Separating arterioles from venules from dextran-TRITC tracer kinetics**

To separate arteries from veins in the murine neocortex, we intravascularly perfused dextran-TRITC via tail vein injection. The dynamics underlying indicator dilution curves has been well studied and is modeled after the gamma-variate function (Eq. 10) (THOMPSON et al., 1964). The gamma-variate function is parameterized by 4 variables – amplitude ($A$), time to initial response ($t_0$), shape-parameter ($r$), and scale-parameter ($b$).

$$G(A, t_0, r, b) = \begin{cases} A(t - t_0)^r e^{-\frac{(t-t_0)}{b}}; & t \geq t_0 \\ 0; & otherwise \end{cases}$$ \hspace{0.5cm} (10)

After performing a 1.5 Hz lowpass filter on the dex-TRITC time courses, we imported the image stack into AFNI and utilized 3dNLfim (https://afni.nimh.nih.gov/pub/dist/doc/program_help/3dNLfim.html) to find the best-fit parameters for the gamma-variate function. $R^2$ allowed us to access the goodness of
the curve fit. The time to peak map, taken as the maximum of \( G \), used in conjunction with custom software developed in MATLAB to annotate vessels (Senarathna et al., 2017), allowed us to separate the arties from veins.

**Statistical analysis**

The \( t \)-statistic was used to test for significance of both cross-correlation values observed in resting state connectivity map, and the \( 1/f \) coefficient in the non-linear regression model presented in Eq. 5. Significance was set a \( p < 0.001 \) with Bonferonni correction.

### 3.3 Results

#### 3.3.1 Benchtop optical imaging system was able to capture microvascular resolution images of the whole-murine brain

The benchtop optical imaging system was capable of imaging the whole murine-brain at microvascular spatial resolution. The system setup can be seen in **Fig 3.1a**. A white light source with a color filter wheel allowed us to perform optical intrinsic signal (OIS) imaging. The 632nm red laser allowed us to perform laser speckle contrast imaging (LSCI). The green laser allowed us to capture dex-TRITC tracer kinetics. Collectively, this information was captured by a 16-bit CCD camera, and sent to a master program in MATLAB. **Fig 3.2b (left)** presents an example cerebral blood volume (CBV) map from a tumor-bearing mouse acquired from OIS imaging at 570nm. **Fig 3.2b (right)** presented
an example cerebral blood flow (CBF) map from a tumor-bearing mouse acquired from LSCI processing (Methods).

Fig. 3.1: Benchtop optical imaging system was able to acquire microvascular resolution images of the whole-murine brain. (a) Schematic of the multi-modal benchtop optical imaging system. (b) (left) Whole brain map acquired from 570nm OIS green light absorption imaging, capturing total oxyhemoglobin (HbT), or, equivalently, cerebral blood volume (CBV). (right) Whole brain map acquired from 632 nm red laser and subsequent laser speckle processing, capturing cerebral blood flow (CBF). Presented hemodynamic maps were acquired from a brain tumor bearing mouse during the resting state imaging protocol.

3.3.2 Registration of murine 2D neocortex atlas to mouse subject and region of interest segmentation

Using a 2D hierarchical murine neocortex atlas, we were able to register and segment various functional ROI on the murine brain. Fig 3.2a presents the atlas with ROIs shown in color. We then registered the atlas to the mean CBV map acquired from resting state imaging (for example, see Fig 3.1b, left) using a landmark based method.
(Methods). An example registered atlas is shown in Fig 3.2b. Lastly, using Amira, we manually segmented the various ROIs on the murine skull (Fig 3.2c).

Fig. 3.2: Registration of murine 2D neocortex atlas to mouse subject and region of interest segmentation. (a) 2D atlas of the murine neocortex (Allen Brain Atlas). (b) Transformed and cropped atlas after registration to mean cerebral blood volume (CBV) map acquired during resting state protocol. (c) Regions of interest (ROIs) overlaid on CBV map, acquired from manual segmentation after atlas registration.

3.3.3 Visualization of brain tumor-induced alterations in resting state connectivity – initial findings

Figs 3.3a,d shows the segmented ROI label fields for an example healthy and brain tumor bearing mouse, respectively. Figs 3.3b,e show the left somatosensory (left white arrows) resting state connectivity maps for healthy and tumor-bearing subject, respectively, acquired from measurement of CBF dynamics. Here, one can observe that the percentage of pixels which were significantly correlated or anti-correlated decreased in the tumor bearing brain, mostly in contralateral brain regions (Fig 3.3e).
Figs 3.3c,f show the left somatosensory resting state connectivity maps for healthy and tumor-bearing subject, acquired from measurement of CBV dynamics. In Fig 3.3c, one can observe the bilateral symmetry between left somatosensory cortex and right somatosensory cortex (right white arrows). This symmetry is lost due to the presence of brain tumor (Fig 3.3f). CBV maps are used as underlay in all images to highlight the vascular topology.

**Fig 3.3: Visualization of brain tumor-induced alterations in resting state connectivity** (a) Healthy brain label field overlaid on a map of cerebral blood flow (CBV), showing left somatosensory cortex (left white arrow) and right somatosensory cortex (right white arrow). (b) Resting state connectivity map for left somatosensory cortex acquired from measurement of cerebral blood flow (CBF). (c) Resting state connectivity map for left somatosensory cortex acquired from measurement of CBV. (d) Tumor-bearing brain label field overlaid on a map of CBV, showing left somatosensory cortex (left white arrow) and right somatosensory cortex (right white arrow). Tumor is located in left somatosensory cortex. (e) Resting state
connectivity map for left somatosensory cortex acquired from measurement of CBF. (f) Resting state connectivity map of left somatosensory cortex acquired from measurement of CBV. Presented connectivity maps are thresholded at $p < 0.001$ with Bonferroni correction.

3.3.4 Whole-brain disruptions of resting state connectivity induced by tumor – initial findings

Here, we observed that CBF and CBV dynamics were altered by the presence of brain tumor, though not necessarily equivalently. Fig 3.4b presents the resting state connectivity matrix for a healthy subject acquired from measurement of CBF. We observed strong bilateral symmetry in visual and auditory regions, but weak motor and somatosensory connectivity. In addition, ipsilateral auditory-visual connectivity appeared to be strong in both hemispheres. With the presence of brain tumor (Fig 3.4a), we observed that the bilateral auditory and visual networks were attenuated as well as ipsilateral auditory-visual network in the left hemisphere. However, auditory-visual connection in the right hemisphere remained in-tact. The difference matrix can be seen in Fig 3.4c.

Fig 3.4e presents the resting state connectivity matrix for healthy subject acquired from measurement of CBV. These findings are very similar to Fig 3.4b, except we also observed stronger motor and somatosensory bilateral connectivity (Fig 3.4e) that were attenuated by the tumor (Fig 3.4d). In the presence of a tumor (Fig 3.4d), the auditory and visual bilateral networks also appeared to be attenuated, like in Fig 3.4a.
Interestingly, the connectivity in auditory-visual and primary-secondary motor regions increased in both hemispheres. **Fig 3.4f** presents the ‘difference’ matrix.

**Fig 3.4: Whole-brain disruptions of resting state connectivity induced by tumor – initial findings.**

Correlation coefficient (CC) matrices illustrating the resting state connectivity for (a) ROIs from brain tumor-bearing mouse; (b) ROIs from a healthy mouse. (c) The ‘difference’ matrix between tumor-bearing and normal mouse ROIs illustrating tumor induced alterations in resting state connectivity. (a-c) Were acquired from measurement of cerebral blood flow (CBF). (d-f) are equivalent to (a-c) except were acquired from measurement of cerebral blood volume (CBV).

### 3.3.5 Temporal-frequency analysis reveals temporal instability in tumor region compared to contralateral hemisphere – initial findings

Acquisition of 15-20 minutes of cerebrovascular dynamics in the resting state allowed us to probe the temporal-frequency relationship between tissue affected by
brain tumor and healthy brain region in contralateral hemisphere. In this single subject study, we observed that tumor region appears to exhibit considerably higher temporal instability relative to contralateral hemisphere in both CBF and CBV dynamics. Fig 3.5a shows the tumor-GFP signal with the tumor location shown by the left white-hatched outline. The contralateral/control region is shown by the right white hatched outline. Plots over a 4-minute window from the tumor and contralateral dynamics show that tumor region qualitatively exhibited higher variance when CBF (Fig 3.5b) and CBV (Fig 3.5c) dynamics were measured. Spectrograms were then computed (Methods) over the entirety of the imaging session to visualize the temporal dynamics of the frequency components. Figs 3.5e,f present the spectrogram for CBF measurement rom tumor and control tissue, respectively. Additionally, Figs 3.5h,i present the spectrogram for CBV measurement from tumor and control tissue, respectively. We observed not only higher power in tumor tissue region for both CBF and CBV dynamics, but also noticed that power contribution across frequency elements changed over time (Figs 3.5e,h). This can also be observed from the CBF and CBV periodograms (Figs 3.5d,g, respectively). The larger error bars shown in the tumor curves (orange) relative to the control curves (blue) are an indication that frequency power contributions fluctuated more in the tumor tissue. Lastly, the frequency distribution for both regions follow the canonical 1/f curve, however the best-fit coefficient was higher in tumor tissue (Figs 3.5d,g dashed curves). This may be related to the significant metabolic demands of brain tissue immediately surrounding the brain tumor.
**Fig 3.5: Temporal-frequency analysis reveals temporal instability in tumor region compared to contralateral hemisphere – initial findings** (a) GFP signal indicating location of brain tumor. Left hatched outline indicates tumor region and right hatched outline indicates contralateral hemisphere. Plots of tumor (orange curve) and contralateral (blue curve) hemodynamic waveforms over a four-minute epoch after bandpass filtering, smoothing, and global signal regression; (b) cerebral blood flow (CBF) and (c) cerebral blood volume (CBV). (d) Power spectral density estimate of tumor ROI (solid orange curve) and contralateral ROI (solid blue curve) time courses arrived at via Welch’s method. (Methods) of the spectrograms presented in (e) and (f), respectively. Curves are plotted as mean +/- standard deviation. Also shown in (d) are the nonlinear fits of mean tumor ROI periodogram (dashed orange curve) and contralateral ROI periodogram (dashed blue curve) to Eq. 5 alongside their respective equations and R².
values. \((d-f)\) were acquired from measurements of CBF while \((g-i)\) is the CBV equivalent to \((d-f)\). The t-statistic of the 1/f coefficient in the non-linear regression model (Eq. 5) allowed us to access if measured resting state temporal dynamics is congruent with current theory. \((d, \text{tumor}) t = 5.39 (df = 18; p < 0.001); (d, \text{contralateral}) t = 5.1 (df = 18; p < 0.001); (g, \text{tumor}) t = 8.08 (df = 18; p < 0.001); (g, \text{contralateral}) t = 8.56 (df = 18; p < 0.001)\)

**3.3.6 Entropy metric appears to be a useful tool for isolating tumor affected tissue – initial findings**

CBV dynamics in response to carbogen varies greatly in a brain-tumor bearing mouse. In addition, the parameters of the HRF may be combined to create a single-snapshot of brain tumor affected tissue. To illustrate this, we performed HRF modeling (Methods) between the carbogen box-car stimulus and measured CBV waveforms. **Fig 3.6a** shows the goodness-of-fit of the model for a healthy (left) and a tumor-bearing (right) subject. Interestingly, the model was not a very good indicator of CBV dynamics in the right hemisphere of the healthy mouse brain (**Fig 3.6a, left**). Nonetheless, using the percent change from baseline, we present a snapshot of a tumor-bearing murine brain during carbogen inhalation (**Fig 3.6b, left**). Clearly, one can see the dramatic decrease in CBV, implying vasoconstriction, as shown by the white arrow. After modeling, we noticed that the parameter maps of the second-gamma variate function (Methods) were heterogeneous. **Fig 3.6b, center** shows the \(r\)-map and **Fig 3.6b, right** shows the \(b\)-map. White arrows point to regions of heterogeneity and are spatially positioned in brain tissue similar to the white arrow in **Fig 3.6b, left**. Knowing this, we wanted to combine the two parameters into a single metric in order to capture brain
tumor affected tissue. To do this, we calculated the pixel-wise ‘entropy’ (Methods) of the second gamma-variate function, which utilized both the shape and scale parameters. Presented in Fig 3.6c are the entropy maps for a single healthy (left) and tumor-bearing (right) subject. The black hatched outline in Fig 3.6c, right highlights brain tissue potentially affected by the presence of brain tumor. Such a hotspot was not noticed in the healthy entropy map (Fig 3.6c, left). While the spatial scope of brain-tumor affected tissue shown in Fig 3.6b, left and Fig 3.6c, right were nearly identical, the entropy map (Fig 3.6c, right) was able to identify a potential feeding vessel more clearly than using percent change from baseline. Lastly, we wished to visualize the HRFs in various regions of the brain tissue as indicated by the entropy map of a tumor-bearing subject (Fig 3.6c, right). The red ROI indicates potential tumor-affected tissue and the blue ROI is contralateral tissue (Fig 3.6c, right). Plots of the averaged HRF found in these ROIs (Fig 3.6d) show that tumor tissue (red curve) had significantly different cerebrovascular dynamics relative to control/healthy tissue (blue curve) in response to a vasodilatory agent. For example, in healthy tissue (blue curve), there is a brief initial dip followed by long period of rising CBV. In contrast, the HRF from potentially tumor-affected tissue (red curve) showed the opposite dynamics with higher magnitude. Specifically, there was an initial sharp increase in CBV followed by dramatic and sustained period of decreasing CBV.
Fig 3.6: Entropy metric appears to be a useful tool for isolating tumor affected tissue – initial findings
(a) $R^2$ maps indicate how well the best-fit HRF convolved with box-car carbogen input fit the original hemodynamic waveform in healthy subject (left) and tumor-bearing subject (right). (b) Minimum intensity projection (MIP) of cerebral blood flow (CBV) percent change relative to baseline during carbogen inhalation for tumor bearing subject (left). r-parameter (center) and b-parameter (right) maps acquired from modeled HRF's second gamma-variate function (Eq. 6). White arrows in (b) point to possible tissue affected by tumor. (c) Entropy map for healthy subject (left) and brain tumor-bearing subject (right). Black hatched outline indicates abnormally high entropy. (d) Best-fit HRF waveforms plotted for an ROI in contralateral brain (blue curve) and ROI that may be affected by tumor (red curve). Red and blue boxes in (c, right) correspond to (d). HRF modeling was performed on CBV response.

3.3.7 Tracer kinetic modeling enables arteriole and venule separation

Intravascular perfusion of dex-TRITC allowed us to differentiate arterioles from venules. Accessing the time to peak allowed us to perform this differentiation. The time course of the tracer kinetics can be observed in Figs 3.7a-d. For example, at 3.5 s post injection (Fig 3.7b), we can qualitatively identify arterioles (white arrow) while at 5.0 s
post injection (Fig 3.7c), we can qualitatively identify venules (white arrows). To properly acquire the time to peak, we modeled the tracer kinetics as a gamma-variate waveform through the AFNI software (Methods). Fig 3.7e presents a CBV map with green crosshairs pointed to a 7x7 ROI. The waveforms in this ROI are shown in Fig 3.7f, where black curves present original dex-TRITC waveforms after 1.5 Hz lowpass filtering. The red curves represent reconstructed waveforms after modeling, and are overall a good fit to the original data. From the gamma-variate model, we acquired a map of the gamma-variate time to peak (Fig 3.7g). Vessels were then annotated (Fig 3.7h), and in conjunction with Fig 3.7g, a vessel mask identifying arteries, veins, and skull vessels was produced (Fig 3.7i).
Fig 3.7: Tracer kinetic modeling enables arteriole and venule separation. (a-d) Time evolution of dex-TRITC kinetics to highlight the temporal diffusion evolution across the cerebrovascular network. White arrows point to vessels that are perfused at different times. (e) Cerebral blood volume (CBV) map with green crosshairs. (f) 7x7 pixel window (corresponding to crosshairs in (e)) displaying original dex-TRITC waveforms (after 1.5 Hz lowpass filtering) (black curves) with gamma-variate fit overlaid (red curve). $R^2 > 0.95$ was recorded for all pixels. (g) Time to peak map of the modeled gamma-variate functions. (h) Vessel annotation map. Using these, we acquired a mask of (i) arteries, veins (blue), and skull vessels (black). (h) Used with permission from Senarathna et al (Senarathna et al., 2017).
3.4 Discussion

Imaging techniques that can capture neurogenic vascular response have become useful tools for investigating brain functionality. To date, however, the consequences of brain tumor-induced neurovascular uncoupling on microvascular hemodynamics is poorly characterized; understanding this may yield greater insight towards characterizing cognitive side-effects and in developing more thorough treatment options. Therefore, we hypothesized that microvessel-scale optical imaging measurement of cerebral blood flow (CBF) and cerebral blood volume (CBV) could be used to detect perturbations in resting state fluctuations and vascular functionality of the murine neocortex arising from brain tumor-induced NVU.

Visualization of resting state connectivity maps showed that the presence of a brain tumor in the left somatosensory cortex can disrupt connectivity to contralateral hemisphere. Overall, these maps combined with cross-correlation matrices allowed us to observe attenuation in bilateral resting brain networks. Additionally, CBF measurement showed disruptions in networks in hemisphere ipsilateral to the tumor. Spectrogram analysis of the superficial brain tissue above the brain tumor ROI showed a highly perturbed environment where both CBF and CBV dynamics exhibited non-stationary temporal-frequency properties, relative to contralateral brain tissue. To characterize whole-brain vascular functionality, we subjected the mice to carbogen inhalation. By modeling the hemodynamic response function (HRF), we observed significant differences in the transfer function dynamics, responsible for modulating
vascular tone, between superficial brain tissue surrounding brain tumor and contralateral brain. This allowed us to utilize an ‘entropy’ metric to isolate potentially tumor-affected tissue. Lastly, using intravascular perfusion dynamics of dex-TRITC, we could differentiate arteries from veins. While the study presented is currently limited by sample size, we have established a platform to delve into tumor induced disruptions of cerebrovascular dynamics at the level of individual vessels.

Resting state measurement of hemodynamic variables acquired from optical imaging has reported bilateral networks in the murine neocortex (Bergonzi et al., 2015; White et al., 2011). While we reported similar findings, the resting state connectivity maps of the left somatosensory cortex presented highlight that CBF and CBV dynamics did not yield equivalent network structures. One reason for this may be due to the high noise associated with LSCI (Senarathna et al., 2013), which may be reduced by increasing the spatial-window radius seed for LSCI preprocessing. Additionally, the underlying mechanism behind CBV and CBF fluctuations, while often coupled (Roy and Sherrington, 1890), are not necessarily synchronized. Collectively, this leads to the possibility that contributions from non-neurovascular coupling sources, such as autoregulatory vasomotor waves (Kiviniemi, 2008), lead to non-linear modulation in local CBF and CBV.

Delving further, current literature using microvascular optical imaging to map resting state connectivity in the murine brain does not take advantage of the microvascular spatial resolution. Specifically, most processing steps to date yield effective resolution on par with that of fMRI acquisition (Bergonzi et al., 2015; White et
al., 2011). Our motivation for presenting results at a similar resolution was to first see if we could obtain congruent results. Using this processing schema, we discovered that a brain tumor located approximately in the left somatosensory cortex disrupted bilateral connections in somatosensory, auditory, and visual networks. Additionally, CBF measurement showed that auditory-visual connection in the hemisphere ipsilateral to the tumor was disrupted as well. It was unclear why this was not also seen in CBV measurements and is an ongoing area of study in our lab.

As this work evolves, we will utilize a novel preprocessing step to reduce noise while preserving spatial resolution (Senarathna et al., 2017). Analysis by Senarathna et al has already sought to characterize the resting state connectivity contributions of individual arterioles and venules (Senarathna et al., 2017). We will pursue this same analysis to characterize tumor induced alterations in individual vascular resting state dynamics.

In dissecting measured CBF and CBV resting state dynamics, we sought to characterize the temporal-frequency dynamics in brain tissue above brain tumor and contralateral brain. One hypothesis for the observed phenomena may lie in the underlying cellular energy metabolism, which is often affected during the transition from normal to cancer cells (Marie and Shinjo, 2011). For example, brain tumors often use glycolysis as a main source of energy, despite the presence of oxygen (Warburg, 1956). Overall, the combination of abnormal vasculature (Kim et al., 2011) and flow profile (Jain et al., 2007) may mediate vascular steal in order to maintain operational
metabolic levels. This may have resulted in increased variance in flow and volume in brain tissue surrounding the tumor.

Interestingly, we found that both ROIs in this analysis exhibited the canonical 1/f frequency distribution (Cordes et al., 2001). It would be interesting to see how this relationship is affected by neurovascular uncoupling (NVU). To test this, we are in the process of performing histology to find evidence of disruptions in the neurovascular unit. It would also be worthwhile to perform dual LSCI/OIS imaging and EEG for additional validation. For example, neuronal activation with a lack of vascular response would implicate NVU.

Current work using MRI has studied tumor vascular status on carbogen-induced oxygenation (Chakhoyan et al., 2017; Lanzen et al., 1998). Using brain tumors, Chakhoyan et al. found that CBV increases in peritumoral and tumor region were largely dependent on the capacity to vasodilate (Chakhoyan et al., 2017). In contrast, our carbogen study showed significant decreases in cerebral blood volume in tissue nearby the brain tumor inoculation site, implying vasoconstriction. One reason for this may be that the location of tumor relative to superficial tissue. Because we were unable to image the tumor-GFP signal in this example, it is possible that the tumor position was quite deep. Another possibility may arise from disruptions in autoregulation. Research using stroke models found lower amplitude and longer time to peak in the HRF (Altamura et al., 2009; Lindauer et al., 2010), reflecting disrupted cerebral autoregulation. Similarly, we observed a dramatic difference in the ‘tumor-region HRF’ relative to the HRF representing contralateral brain. While the nature of the changes is
not on par with that observed by Altamura et al (Altamura et al., 2009) (i.e. we observed increased amplitude and shorter time to peaks), nonetheless further investigation in understanding the underlying principal for these deviations may be important for diagnostic and treatment efficacy.

Lack of a tumor-GFP signal limited us from confirming if the ROI showing dramatic CBV decrease was above the brain tumor. Having this evidence would be extremely useful not only to confirm tumor position, but also to quantify the overlap between tumor-GFP signal and the calculated tumor ‘entropy’ map. Precise tumor location would also be extremely useful for quantifying deviations in the functional HRF as a function of distance. Nonetheless, our initial findings of a significant region of increased ‘entropy’ combined with contrasting HRF relative to contralateral brain holds promise for probing alterations in vascular functionality as induced by brain tumor.

One downside to our study comes from our whole-brain preparation prior to imaging. Saline leakage from the cranial window affected our ability to acquire image stacks for long periods of time (~ hours), and is the reason for the limited sample size. Efforts are currently underway to resolve this. Secondly, the position of tumor cells was often times too deep to capture tumor-GFP signal. This led us to performing a time-consuming brain clearing procedure which is still ongoing. Due to these limitations, we do not have tumor-GFP signal for the tumor-bearing mouse presented in the carbogen analysis as mentioned above. Most importantly, it will be necessary to provide evidence of NVU in order to justify our study. As previously mentioned, we are currently in the process of histological validation to show disruption of the neurovascular unit. We are
also investigating the option of dual optical imaging and EEG for further validation. However, because we do not have data for either of these presently, we cannot conclude if NVU was involved in the results presented above. Nonetheless, it would be interesting to see what results presented above hold in cases of both intact and disrupted neurovascular coupling. Such findings may allow us to develop generalizations inherent to all brain tumors while also probing deeper into changes solely induced by NVU.

Although preliminary, these studies lay the foundation for techniques to better characterize the impact of NVU on cerebrovascular dynamics at both the local and global scale.
Chapter 4: Additional Applications of Functional Optical Imaging

Here, we shift from the topic of tumor modulations of resting-state cerebrovascular dynamics to stimulus-modulated changes. We will demonstrate the utility of multi-modal functional optical imaging for developing a comprehensive understanding of brain functionality in response to a stimulus or task. To investigate this topic, we utilized a novel miniaturized microscope developed in our lab with multiple features. These include excellent spatio-temporal resolution, multi-contrast imaging capabilities that can acquire both neuronal and hemodynamic variables, and the opportunity to perform studies in awake, freely behaving animals. Specifically, we present a wide-field functional optical imaging study of the murine auditory cortex. (N.B. The work presented here is a part of a manuscript that is currently in submission, to which I contributed to the data processing and modeling aspects).


4.1 Introduction

A wide variety of techniques have been employed to characterize the highly complex and dynamic cortical processes underlying sensory perception. For example, platforms such as electrocortiography (ECoG) can yield direct evidence of neuronal activation through the measurement of neurogenic local field potentials or LFPs (Buzsaki et al., 2012). Despite the pivotal role ECoG has played in mapping cortical regions
associated with speech (Taplin et al., 2016) and motor actions (Zhang et al., 2010), amongst others, and its use in studies of neuronal dysfunction such as epilepsy (Palmini, 2006; Yang et al., 2014), it is limited in spatial resolution (∼ cm) and, more importantly, hampered by the uncertainty that results when trying to record LFPs from a heterogeneous medium like the skull and brain (Buzsaki et al., 2012). To circumvent these issues, neuroimaging techniques such as blood-oxygen-level-dependent (BOLD) functional MRI (fMRI), which takes advantage of neurovascular coupling (Roy and Sherrington, 1890) and the paramagnetic properties of blood (Ogawa et al., 1990), have been utilized to map functional areas of the brain. However, the drawback of fMRI is its low temporal resolution and reliance on changes in cerebral blood flow as a surrogate marker of brain activation. Current literature suggests the use of a ‘hemodynamic response function’ to capture cerebrovascular dynamics in response to a stimulus (Buxton et al., 2004). Traditionally, fMRI techniques assume a simplified relationship between the ‘box-car’ stimulus model and the underlying neural dynamics (Friston et al., 1995) . Collectively, this approach can result in lack of statistical power, bias, and parameter confusability (Lindquist et al., 2009). Therefore, a neuroimaging technique that can directly capture neuronal activation along with vascular dynamics at high spatio-temporal resolution would be an important step towards a more reliable platform for understanding brain function and dysfunction.

Functional optical imaging has brought significant opportunities for neuroscientists to visualize the multi-faceted nature of brain function (Ma et al., 2016; Sun et al., 2014). The availability of high speed, microvascular spatial resolution image

81
acquisition makes this platform suitable for acquiring neuronal and vascular dynamics. In addition, miniaturization makes it possible for brain processes to be interrogated in awake animals, freeing researchers from the burden of having to study the brain under unnatural conditions such as anesthesia. However, most current miniaturized optical imaging instruments lack the ability to simultaneously interrogate vascular (Miao et al., 2011) and neuronal (Ghosh et al., 2011) dynamics. To fulfill this unmet need, our lab developed a miniaturized microscope (Senarathna, 2017) that combines three optical contrast mechanisms - fluorescence (FL) (Osman et al., 2012), hemoglobin intrinsic optical signals (IOS) (Hillman, 2007), and laser speckle contrast (LSC) (Senarathna et al., 2013). The FL channel enables imaging of neural activity with genetically encoded calcium indicators such as GCaMP (Lin and Schnitzer, 2016). LSC can be utilized to measure cerebral blood flow, an important variable that is directly influenced by neuronal activation and the neurovascular coupling mechanism. Cerebral blood volume, a variable that is tightly coupled with cerebral blood flow, can be measured through the IOS channel. In addition, the microscope is well-suited for awake animal studies, has a spatial resolution of 5 μm, a FOV of 3 × 3 mm², and acquires images at 15 frames per second (fps). Utilizing this novel technology at its full capacity, here we investigated cortical activation in response to a stimulus (Senarathna, 2017).

Specifically, we conducted a multi-contrast optical imaging study of the murine auditory cortex in response to sound stimuli in an awake mouse. Using the three imaging channels mentioned, we acquire neuronal and vascular dynamics in response to 4 kHz and 24 kHz auditory stimulation. First, to showcase the microscope’s capabilities,
we present high resolution optical images acquired through the LSC and IOS channels. Next, having presented a mouse with an auditory stimulation, we show how the microscope was capable of capturing neural activation and the concomitant functional hyperemic response in the auditory cortex. Lastly, we utilized these neurovascular variables to perform wide-area functional mapping using two distinct models of activation. The first model utilizes the frequency specific neuronal activation to compute a spatially specific vascular response, while the second model uses the canonical hemodynamic response function to generate maps of salient physiological variables such as firing rate, onset latency, and duration of activation. Collectively, we show the importance of interrogating both the ‘neural’ and ‘vascular’ aspects of brain function, and the increasing role of preclinical multi-contrast optical imaging studies in the field of neuroscience.

4.2 Methods

4.2.1 Cranial window preparations

Anesthesia was induced with 4% isoflurane at an O₂ flow rate of 0.5 L/min. The mouse head was secured to a stereotaxic frame with a bite bar while anesthesia was maintained at 1-2% isoflurane with 0.5 L/min through a tube attached to the bite bar. Petroleum jelly (Vaseline) was spread over the mouse’s eyes to prevent dehydration. The hair over the scalp was trimmed and the exposed area sterilized with 70% ethanol. Injections of dexamethasone (2 mg/kg) and carpofren (5 mg/kg) were administered i.p. After lidocaine (2% with 1:100,000 epinephrine) was injected locally, an incision was made on the midline of the head. The skin and fascia were removed over the auditory
cortex. The area was thoroughly dried and a primer (Optibond, 33533) applied to the surface. A custom-built steel head post was secured with a UV-cured dental cement (Heraeus, 0197) to the exposed area of the skull. Using vascular and local landmarks, the area of the cranial window (above the left auditory cortex) was identified with a pen. A craniotomy was then performed with a microdrill (Fordom, MH-170) and the bone detached from the skull leaving the dura intact. After clearing the area of debris, a cover-slip was placed on top of the craniotomy and secured with self-curing dental cement (Parkell, S380). During the procedure, 0.9% saline was injected into the scruff of the neck to prevent dehydration. After the procedure was finished, buprenorphine (.5 mg/kg) was injected i.p. (analgesic) and Bacitracin (Fougera) administered topically.

4.2.2 Experimental Procedure

A cranial window was prepared over the left auditory cortex of a female tetO-GCaMPs×CaMKII-tTA mouse (Wekselblatt et al., 2016). Next, a head post was surgically attached on the mouse skull. After complete recovery from surgery and habituation to the imaging setup (~ 2 months), the mouse was anesthetized by isoflurane inhalation in a chamber, and subsequently its head immobilized in the imaging apparatus. The mouse remained awake for the entire duration of the imaging experiment. Imaging runs were split into sixty epochs of 10 s each (i.e. total imaging duration = 10 min) and images acquired under blue light illumination at an exposure time of 50 ms at 15 fps. Within each epoch, a 300 ms auditory stimulus was presented at the 3s time point. The auditory stimulus was randomized to be either a 4 kHz or 24 kHz tone. A total of 30 stimulus presentations for each 4 kHz and 24 kHz tone were conducted. This procedure
was then repeated under green light and red laser illumination. The green light and laser imaging were done at 50 ms and 10 ms exposure times, respectively. The frame rate remained ~15 fps. The auditory stimulus generated a sync pulse at the beginning of each 10 min trial. This enabled the microscope data acquisition and the stimulus presentation to be time-locked. Each image acquired by the microscope was time-stamped at ms temporal resolution.

### 4.2.3 Image processing

**Laser speckle contrast**

LSC was calculated by processing a stack of red laser images. The speckle contrast \( k \) at each pixel \((x, y)\) was calculated as (Senaratna et al., 2013):

\[
k(x, y) = \frac{\sigma(x, y)}{\mu(x, y)}
\]

(1)

Here, \( \mu \) and \( \sigma \) are the mean and standard deviation of light intensity at each pixel in the chosen image stack. The image stack size depends on the frame rate and temporal resolution desired for the LSC image. For example, a 4s acquisition at 15 fps, will result in a stack of 60 images.

The speckle contrast \( k \) is related to blood flow speed by (Senaratna et al., 2013):

\[
k^2 = \frac{\tau}{2T} \left\{ 2 - \frac{\tau}{T} \left[ 1 - \exp \left( -\frac{2T}{\tau} \right) \right] \right\}
\]

(2)
Here exposure time is denoted by $T$ and $\tau$ denotes the decorrelation coefficient, a quantity inversely proportional to blood flow speed. For small $\tau$ values (associated with typical microcirculation imaged by our microscope), Eq. 2 can be simplified to:

$$\frac{1}{\tau} \propto \frac{1}{k^2}$$

Eq. 3 was used to compute CBF speed.

*Optical functional imaging experiment*

Images from the last 25 trials (out of 30) for each stimulus presentation were used to calculate the neuronal activation maps. Since the image sensor frame rate can vary slightly, each image was time-stamped at ms temporal resolution. This permitted the acquired images to be resampled to a temporal resolution of 10 ms (Matlab) using linear interpolation. Then, both calcium and hemodynamic responses were calculated as (ImageJ):

$$r(t) = \frac{X(t) - \bar{X}_B}{\bar{X}_B}$$

Here, $r(t)$ is the calculated response, $X(t)$ the measured variable, and $\bar{X}_B$ is the baseline (0 – 3 s) mean of the measured variable. In the case of calcium fluorescence imaging, the light reflectance levels were taken as the measured variable. For HbT and dHb imaging, inverted green light and red laser light images were used respectively, while the relative blood flow levels calculated by LSC were used for CBF. All data were filtered using a 3x3 median filter to reduce noise. The laser illumination based data (dHb and CBF) underwent an additional filtering using a 20x20 pixel mean filter.
**Modeling vascular activation using frequency specific neuronal ‘hotspots’**

The overall signal processing model is schematically shown in Fig. 4.3a, b. Here, the measured hemodynamic signal was modeled as a combination of the cortex wide (global) hemodynamic fluctuations (the ‘background’ time-course), the stimulus specific activation (the ‘activation’ time course), and an error term to account for bias. From this, the spatial distribution of the fit coefficients belonging to the ‘background’ and ‘activation’ signal could be derived, with the ‘activation’ coefficient representing the vascular component of the response.

The hemodynamic data was filtered in the temporal domain using a low-pass filter with a 2 Hz cut-off for noise removal. Next, a ‘hot-spot’ time-course was created by computing the average hemodynamic time-course for a region exhibiting peak neural activity (as identified by a ‘hotspot’ from the maximum intensity projection of the calcium signal image, Fig. 4.3a). The ‘background’ time-course was computed by computing the cortex-wide average. This ‘background’ time-course was then subtracted from the ‘hotspot’ time-course to yield the final ‘activation’ time-course (Fig. 4.3a). The filtered hemodynamic data, together with these two time-courses was input to a least-squares regression model as shown below (Fig.4.3b):

\[ r(t) = c_0 + c_1 a(t) + c_2 g(t) + e(t) \]  \hspace{1cm} (5)
Here, $a(t)$ and $g(t)$ are the ‘activation’ and ‘background’ time-courses, respectively. $c_1$ and $c_2$ are their corresponding ‘coefficients’. $r(t)$ is the measured hemodynamic response, $e(t)$ is the error term and $c_0$ is a constant accounting for any signal offset.

This image processing pipeline was run separately for HbT and CBF signals. The entire procedure was first performed for 4 kHz response data and then repeated for the 24 kHz response data.

*Modeling vascular activation using the canonical hemodynamic response function*

Here, we utilized a gamma-variate function to model, per-pixel, the hemodynamic response transfer function (HRF; Eq. 6) relating the calcium and hemodynamic responses. The HRF function was parameterized by 4 variables – amplitude (A), time to initial response ($t_0$), a shape-parameter ($r$), and a scale-parameter ($b$) as shown below in Eq. 6:

\[
H(A, t_0, r, b) = \begin{cases} 
A(t - t_0)^r e^{-\frac{(t-t_0)}{b}}; & t \geq t_0 \\
0; & \text{otherwise}
\end{cases}
\]  

This approach is widely used in fMRI literature due to the assumed linear relationship between neuronal firing and the concomitant hemodynamic response (Lindquist et al., 2009). Additionally, various physiological interpretations of the gamma-variate waveform (Fig 4.4a, b) are easily accessible. To acquire the optimal HRF, an initial guess for the four parameters was made and optimized via the `fminsearchbnd` function in MATLAB. We assumed that the amplitude parameter must be positive. This optimization technique uses an iterative, derivative free approach to minimize the sum-of-squares.
difference (i.e. maximize the fit) between the hemodynamic waveform and the calcium waveform convolved with the HRF transfer function (Eq. 7).

\[ C(f, H, g) = \sum_{i=1}^{T}(g_i - (H \ast f)_i)^2 \]  

(7)

Here, \( C \) represents the cost-function that is to be minimized, \( g \) is the hemodynamic signal, \( f \) is the calcium waveform, \( H \) is the HRF, and \( \ast \) is the convolution operator. From the optimized parameters, maps of amplitude, time-to-peak, and full-width-half-maximum (FWHM) were computed.

To access how well the reconstructed waveform, taken as the best-fit HRF convolved with calcium dynamics (see Eq. 7), fit the original hemodynamic fluctuations, we utilized the coefficient-of-determination (\( R^2 \)) metric (Eq. 8). \( R^2 \) values close to 1 indicate that the HRF is a plausible model for the underlying neurovascular dynamics, whereas \( R^2 \) values less than 0 suggest that the mean of the original hemodynamic waveform was a better fit than the HRF modeling approach.

\[ R^2 = 1 - \frac{\sum_{i=1}^{T}(y_i - \hat{y}_i)^2}{\sum_{i=1}^{T}(y_i \bar{y})^2} \]  

(8)

Here, \( y \) is the original hemodynamic waveform, \( \hat{y} \) is the reconstructed waveform taken to be the calcium dynamics convolved with best-fit HRF, and \( \bar{y} \) is taken to be the mean value of the original hemodynamic waveform.
4.3 Results

4.3.1 Microscope was capable of acquiring microvascular-scale optical images

A test imaging run on an awake mouse brain shows that the microscope was capable of acquiring high-resolution microvascular images. Here, a mouse fitted with the microscope is shown in an awake state (Fig 4.1a). A representative 572 nm green light absorption image indicative of cerebral blood volume is shown in Fig 4.1b, and cerebral blood flow map acquired after LSC processing is shown in Fig 4.1c.

![Fig 4.1: Microscope was capable of acquiring microvascular level optical images. (a) An awake and freely moving mouse with the microscope. (b) A network of cerebral microvessels visualized with green IOS (GR) illustrating HbT (or CBV) distribution. (c) Pseudo-colored maps of CBF velocities obtained by performing speckle contrast imaging under laser illumination (LSC). Images adapted from Senarathna et al. (Senarathna et al., 2017).](image)

4.3.2 Multi-modal optical acquisition of neurovascular dynamics to auditory stimulation
The neuronal and vascular dynamics of the murine auditory cortex in response to a 4 kHz auditory stimulation (Fig 4.2a) are presented in Fig 4.2. Using GCaMP bound calcium fluorescence (Wekselblatt et al., 2016), we were able to successfully capture evidence of neural activation (Fig 4.2b, green channel). From this image, one can observe two significant calcium ‘hotspots’. Furthermore, utilization of laser speckle and optical intrinsic signal imaging allowed us to capture the functional hyperemic response. For example, acquisition of cerebral blood volume (570nm green light) allowed us to map the vasodilatory response to auditory stimulation across the entirety of the auditory cortex (Fig 4.2b, red channel). Additionally, 632nm laser speckle imaging provided evidence of changes in cerebral blood flow and oxygenation. Altogether, time course analysis of a region of interest lying within one of the neural ‘hotspots’ (Fig 4.2b, green channel) revealed the classic hyperemic lag (~ 1 sec) between neural firing and the onset of hemodynamic response (Hillman et al., 2007) (Figs 4.2c,d,e,f). From these images, we can see that the sharp rise in calcium accumulation from neural firing (Fig 4.2c) was followed by increases in cerebral blood flow (Fig 4.2d) and cerebral blood volume (Fig 4.2e). In contrast, there was a decrease in deoxyhemoglobin concentration within this region (Fig 4.2f). Collectively, the microscope is well suited for capturing the neurovascular dynamics underlying stimulus evoked cortical activation.
Fig 4.2: Multi-modal optical acquisition of neurovascular dynamics to auditory stimulation. (a)

Schematic of the experimental setup for the auditory stimulation and image acquisition. (b) A composite (i.e. RGB) map showing maximum calcium response (green channel) and the corresponding maximum vasodilatory response (red channel) to a 4 kHz stimulus. Anatomical directions dorsal (D), ventral (V), caudal (C), and rostral (R) are marked for reference. Time traces of the calcium and hemodynamic response to a 4 kHz stimulus in the 20x20 pixel region of interest shown in (b). (c) shows calcium dynamics, (d) shows cerebral blood flow, (e) shows cerebral blood volume and (f) shows deoxyhemoglobin fluctuations. The ordinates for each plot are in fractions of the peak response amplitude. The black line indicates the baseline for each trace. Images adapted from Senarathna et al (Senarathna et al., 2017)

4.3.3 Utilizing frequency specific neuronal ‘hotspots’ for wide-area functional mapping of vascular activation
Task or stimulus based functional magnetic resonance imaging (fMRI) techniques (Boynton et al., 1996) have been extensively used over the last two decades to interrogate the functionality of different brain regions (Zuo et al., 2013). Typically, the observed stimulus-evoked blood-oxygen-level-dependent (BOLD) response is input into a pixel-wise regression model to identify activated pixels by removing the effect of time constants (i.e. by deconvolving with an ‘activation’ hemodynamic response function), as well as eliminating the effects of background fluctuations and system noise (Fig. 4.3b).

Here, we processed our multi-contrast imaging data with a standard fMRI signal processing software (Analysis of Functional Neuro Images: AFNI (Cox, 1996)) and implemented an fMRI-type functional mapping of the auditory cortex. This approach is in contrast to conventional functional optical imaging approaches wherein one usually calculates an average signal over the desired FoV without displaying or computing the pixel-wise response.

We treated the calcium response map as the ‘gold standard’ for identifying activated areas, and created an ideal hemodynamic response (i.e. an ‘activation’ time-course) by subtracting the average background hemodynamic signal from the hemodynamic response corresponding to the calcium hotspot pixels (Methods). Fig. 4.3a illustrates this using CBF time-courses. We then input this activation time-course and the background time-course into a linear regression model that generated pixel-wise ‘fit coefficients’ for each time-course (Fig.4.3b and Methods). The ‘fit coefficients’ associated with the activation time-course enabled us to characterize ‘vascular response’ to activation across the entirety of the auditory cortex (Fig. 4.3c). In
accordance with previous tonotopic studies of the murine auditory cortex (Issa et al., 2014), the 4 and 24 kHz calcium activation maps exhibited distinct spatial patterns (Fig. 4.3d, Ca MIP). Following the regression analysis, the positive HbT and CBF activation coefficient maps (Fig. 4.3d, HbT, CBF Act maps) exhibit more tonotopic specificity to the Ca response corresponding to the 4 and 24 kHz stimuli than the maximum intensity projected HbT and CBF maps (Fig. 4.3d, HbT, CBF MIPs).

**Fig. 4.3: Utilizing frequency specific neuronal ‘hotspots’ for wide area functional mapping of vascular activation**

(a) Generation of the ‘activation’ CBF time-course by subtracting the background CBF time-course from the hotspot (i.e. corresponding to the calcium activation pattern) CBF time-course. (b) Schematic of the image processing pipeline. The ‘activation’ and ‘background’ time-courses were used as regressors to compute the activation and background coefficients from the measured signal using a multiple linear regression model. (c) AFNI screenshot showing pixel-wise CBF time courses and their corresponding model fits (black: measured CBF response, red: model fit) over a 9 x 9 pixel area corresponding to the black square in (a). (d) Ca, HbT and CBF maximum intensity projected (2s – 6s) (MIP) and positive activation coefficient (Act) maps.
for 4 and 24 kHz stimuli. All maps were normalized to 0.1%. Two calcium hotspots are marked on each calcium activation map: HS1 and HS2. One can see that the positive HbT and CBF activation coefficient maps exhibit more tonotopic specificity to the Ca signal than the HbT and CBF maximum intensity projected maps. Images adapted from Senarathna et al. (Senarathna et al, 2017).

4.3.4 Wide-field mapping of the hemodynamic response function yields further insight on neurovascular dynamics

We next sought to model the hemodynamic response function (HRF) in order to quantify salient physiological parameters underlying neurovascular coupling. Here, we assumed a linear relationship between neuronal output and concomitant vascular response, such that hemodynamic response results from the convolution of the calcium (neuronal) waveform and a gamma-variate function (4.2 Methods). To date, this approach has been used in fMRI (Colonnese et al., 2008) and optical imaging (Ma et al., 2016) literature.

Fig 4.4 illustrates the output of this modeling approach to 4 kHz auditory stimulation and subsequent cerebral blood volume (CBV) acquisition. Fig 4.4a presents an example gamma-variate function with various parameters highlighted, such as height, time to peak, and full-width half maximum (FWHM). These parameters can be used to generate useful physiological metrics, where firing rate is associated with height, onset latency is associated with time to peak, and duration of activity is associated with the FWHM (Fig 4.4b). The coefficient of determination (R²) map allowed us to access how well the reconstructed hemodynamic waveform (best-fit HRF convolved with calcium signal) fit the original vascular dynamics (Fig 4.4c). Interestingly,
the highest reported $R^2$ values (Fig 4.4c) were found to lie in regions of vascular bed correlated to the 4 kHz CBV MIP image shown in Fig 4.3d. Next, the amplitude, FWHM, and time to peak maps of the HRF function are presented (Figs 4d,e,f). Accounting only for pixels which displayed $R^2$ values greater than 0, one can appreciate the spatial heterogeneity across these physiological parameters. Specifically, the duration of activation across the auditory cortex ranged from several hundred milliseconds to 7 seconds (Fig 4.4e), while the latency of activation was shown to be as high as 1.7 seconds (Fig 4.4f).

Lastly, we sought to visualize the shape of the HRF across various regions of interest (ROIs) of the auditory cortex where $R^2$ values greater than 0 were found. The regions selected can be seen in Fig 4.4g, wherein the blue region is above a neural ‘hotspot’, the orange region lies with in a resolvable vessel, and the gray region lies in a ‘background’ region of the auditory cortex. Fig 4.4h presents the region-averaged HRFs with quantifiable metrics (i.e. height, time to peak, and FWHM) shown in Table 4.1 for each respective HRF. Surprisingly, the blue ROI accounting for the neuronal ‘hotspot’ showed the weakest amplitude response in the HRF, though this ROI overall displayed the shortest delay and lowest dispersion (smallest FWHM). As Fig 4.4b shows, HRF height can be affected by the onset latency and duration of activity despite its expected correlation with neuronal firing rate. Nonetheless, modeling the HRF across the entirety of the auditory cortex allows us to appreciate the spatial heterogeneity of the interplay between neuronal activation and vascular dynamics.
Fig 4.4: Wide-field mapping of the hemodynamic response function yields further insight on neurovascular dynamics (a) An example hemodynamic response function (HRF) modeled as a four-parameter gamma-variate function ($A =$ amplitude; $t_0 =$ time to activation; $r =$ shape-parameter; $b =$ scale-parameter). Extractable metrics, such as height, time to peak, and full-width half-maximum (FWHM) are shown. (b) The relationship between the metrics shown in (a) to underlying physiological variables of importance. Solid lines indicate expected relationships whereas dashed lines indicate relationships that may confound interpretation of the estimated parameters. Image adapted from Lindquist et al (Lindquist et al., 2009). (c) The coefficient-of-determination ($R^2$) map highlighting how well the best-fit HRF model convolved with calcium dynamics (induced through 4 kHz auditory stimulation) fit the measured cerebral blood volume (CBV) dynamics. (d) Spatial map of amplitude acquired from the best-fit HRFs. (e) Spatial map of FWHM acquired from best-fit HRFs. (f) Spatial map of time to peak acquired from best-fit HRFs. (g) Calcium fluorescence maximum intensity projection (MIP) to 4 kHz auditory stimulation. Three regions of interests are shown in the blue, orange, and gray squares. (h) The spatially-averaged HRFs acquired from each region. Pixels with no color overlay in (c) have an $R^2$ values less than 0.
Table 4.1: Spatial heterogeneity in hemodynamic response function manifesting from 4 kHz auditory stimulation

Presented here are the amplitude, time to peak, and full-width half-maximum values from the hemodynamic response functions shown in Fig 4.4h.

<table>
<thead>
<tr>
<th></th>
<th>Region 1 (blue)</th>
<th>Region 2 (orange)</th>
<th>Region 3 (gray)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (a.u.)</td>
<td>0.0017</td>
<td>0.0051</td>
<td>0.0034</td>
</tr>
<tr>
<td>Time to peak (sec)</td>
<td>0.61</td>
<td>0.83</td>
<td>0.81</td>
</tr>
<tr>
<td>FWHM (sec)</td>
<td>1.37</td>
<td>2.26</td>
<td>3.14</td>
</tr>
</tbody>
</table>

4.3.5 Modeling the hemodynamic response function may have limited utility

While the results presented in Fig 4.4 may yield useful insights regarding neurovascular dynamics, we observed that this model may overall have limited utility. Presented in Fig 4.5 are the Ca fluorescence MIP images for 4 kHz (Fig 4.5a) and 24 kHz (Fig 4.5d), as well as the coefficient of determination ($R^2$) maps for 4 kHz CBV (Fig 4.5b), 4 kHz CBF (Fig 4.5c), 24 kHz CBV (Fig 4.5e), and 24 kHz CBF (Fig 4.5f). One can observe that the $R^2$ maps for 24 kHz CBV (Fig 4.5e) and 24 kHz CBF (Fig 4.5f) show $R^2$ values either close to or less than 0 across a significant swath of the auditory cortex. This suggests that there may be a disconnect between the calcium and vascular dynamics,
implying that modeling of the hemodynamic response function may not be appropriate for all situations.

**Fig 4.5: Modeling the hemodynamic response function may overall have limited utility** (a) Calcium fluorescence maximum intensity projection (MIP) to 4 kHz auditory stimulation. (b) Coefficient of determination ($R^2$) map highlighting how well the neuronal dynamics to 4 kHz auditory stimulation convolved with best-fit hemodynamic response function (HRF) fit the original cerebral blood volume (CBV) waveform. (c) $R^2$ map highlighting how well the neuronal dynamics to 4 kHz auditory stimulation convolved with best-fit HRF fit the original cerebral blood flow (CBF) waveform. (d) Calcium fluorescence MIP to 24 kHz auditory stimulation. (e) Same as (b) but for 24 kHz stimulation. (f) Same as (c) except for 24 kHz stimulation. Pixels without an overlay in (b,c,e,f) indicate $R^2$ values less than 0.

### 4.4 Discussion

Here, we exploited the microscope’s high spatial (5 μm) and temporal (15 fps) resolution, and its 3×3 mm² FoV to simultaneously interrogate the entire auditory cortex.
at microvascular spatial resolution in an awake mouse (Figs. 4.2). With the acquisition of neuronal as well as multiple hemodynamic variables, we utilized two modeling approaches to better understand the underlying neurovascular dynamics resulting from a stimulus.

In the first model, we explicitly removed background fluctuations from each hemodynamic response variable, thereby permitting the visualization of more specific functional activation (Fig. 4.3c). We were able to demonstrate the tonotopic specificity of the hemodynamic responses to 4 and 24 kHz auditory stimuli (Fig. 4.3d HbT, CBF) by using the distinct spatial calcium dynamics (Fig. 4.3d, Ca) as the gold standard of tonotopic activation. Interestingly, while hemodynamic specificity was observed, the spatial extent of vascular activation seen in both the CBV and CBF activation maps was not constrained to the neuronal hotspots. This suggests that researchers utilizing biomedical imaging modalities that cannot measure direct evidence of neuronal activation, such as fMRI, will need to be cautious when correlating measured responses to the site of activation.

In the second approach, we sought to model the canonical hemodynamic response function (HRF). This allowed us to acquire a multi-dimensional map of important physiological variables, such as response amplitude, onset latency, and activation duration (Fig 4.4). HRF modeling of CBV dynamics in response to 4 kHz auditory stimulation appeared to result in moderate to high value fits across the FoV. Furthermore, the range of values for the extractable HRF metrics were in line with those reported in literature (Hillman, 2014). The onset latency and width of activation
extracted from the HRF of the neuronal hotspot region (blue) were observed to be shorter than the other regions. This is congruent with fMRI literature which correlated reduced onset and width values to robustness of neurovascular coupling (Colonnese et al., 2008). However, as mentioned previously, we observed that the HRF taken from this hotspot region had the lowest amplitude. Lindquist et al. found in stimulation studies that the gamma-variate model gives reasonable results for onsets and widths up to 3 seconds, and underestimates amplitude dramatically at higher onset and width (Lindquist et al., 2009). However, the associated onset latency and duration of activation were well below these limits for the HRF in question. The incongruity may be more easily resolved if we consider the original 4 kHz CBV and CA MIPs. Specifically, the amplitude of the HRF transfer function will be highest where the CBV MIP is greatest and Ca MIP is lowest. While the above interpretation by Lindquist et al. (Lindquist et al., 2009) may be suitable for fMRI resolution modeling, acquisition of neuronal and hemodynamic waveforms at a scale where individual microvessels can be resolved may require its own special considerations.

In contrast to the first modeling approach which achieved high $R^2$ fits (data not shown) across the entirety of the auditory cortex, such was not the case when utilizing the HRF. This is especially evident for the CBV and CBF acquisition to 24 kHz stimulation (Fig 4.5). There are a few reasons why this may be. Whereas in the first model we explicitly modeled each pixel as a combination of a global or background signal and activation dynamics, in the HRF approach, we did not regress the global signal for the final analysis. In an analysis where we did such a regression (data not shown), we
observed less satisfying results as defined by the goodness of fit statistics. This may be due to the presence of stimulus induced signal in the global signal. Overall, the poor fit we observed as we moved farther from the location of neuronal hotspots may be due to a change in the ratio of resting state vascular dynamics and stimulus-induced signal. In pixels where the stimulus-induced signal is negligible, there may be a disconnect between the neuronal dynamics acquired through fluorescence imaging and hemodynamic variables acquired through either LSCI or OIS. In that case, a gamma-variate function would not be an appropriate model of the relationship between these two processes, or the assumption of a linear relationship between the two may be invalid. However, further studies will be necessary to draw such conclusions.

This multi-contrast approach to functional imaging wherein both, the neural and hemodynamic responses can be characterized simultaneously and independently has the potential to improve and inform image processing strategies for other imaging modalities. The micron-scale resolution at which optical imaging is capable of operating allows us to mark individual arteries and veins, both of which exhibit their own dynamics in response to activation. Therefore, we note that direct translation of fMRI approaches to optical imaging will need special considerations. Nonetheless, our demonstration can serve as a model for microscopy image processing and provide a data analysis framework for future researchers.
Chapter 5: Future Directions

We envision that MRI will continue to play a singular role in neuroscience research. As has been previously mentioned, brain tumor induced neurovascular uncoupling (NVU) often confounds the interpretation of clinical fMRI (Agarwal et al., 2016). Overall, this obscures the extraction of neuronal information that is essential for evaluating the effect of GBM progression on eloquent cortical areas. To that effect, we investigated this phenomenon to better characterize the global impact of brain tumor development on the rsfMRI BOLD signal. The findings we have presented have opened up several avenues of investigation. For example, it still remains to be seen whether “normalizing” the brain tumor vasculature by suppressing growth of angiogenic vessels with antiangiogenic therapies can reverse the effects of tumor induced neurovascular uncoupling (NVU) (Jain, 2001; Jain et al., 2007). If NVU can be reversed, then rsfMRI has the potential to detect this recovery.

Additionally, functional optical imaging offers a range of research options within and outside the neuroscience domain. The ability to acquire multiple hemodynamic variables such as blood flow and blood volume offers a unique opportunity to interrogate brain disease and rehabilitation at the microvascular scale. For example, optical imaging can allow us to probe deeper into the dysregulation in vessel architecture during tumor angiogenesis (Folkman, 2002), or when the microvascular network is intermittently perfused such as in stroke (Armitage et al., 2010). While the fluorescent contrast mechanism is a desirable means for acquiring neuronal activation
(e.g. via GCaMP), simultaneous recording of ECoG with IOS and LSC channels in applications such as stroke (vasculogenic) and epilepsy (neurogenic) may yield new insights into the etiology of such diseases.

Furthermore, we foresee an opportunity to translate many of the advances in MRI to optical imaging. Recently, Colonnese et al demonstrated that the delayed hemodynamic response in young mice could not be accounted for by the delayed onset of neuronal activation (Colonnese et al., 2008). Using fMRI, the authors showed that the hemodynamic response function changes with age, exhibiting higher amplitudes and delay latencies (Colonnese et al., 2008). However, evidence of functional immaturity of the neurovascular unit was not directly shown. Therefore, a plausible optical imaging experiment one could envision would be to fluorescently label astrocytes (Otsu et al., 2015) to study the longitudinal development of neurovascular coupling, by examining the magnitude response of astrocytic activation in relation to a stimulus. Furthermore, the development of chemical exchange saturation (CEST) MRI (Liu et al., 2013) has provided an opportunity for researchers to investigate cerebral glutamate dynamics in patients with epilepsy (Davis et al., 2015). Results from these studies clearly suggest that in vivo imaging of chemical and molecular signaling is of growing importance for elucidating brain functionality. Since the presence of a fluorescent channel makes fluorescent tagging of a pharmacological agent feasible (Liu et al., 2009), it is possible to utilize optical imaging to investigate the chemical and molecular signaling in epileptic brain tissue in greater depth.
Lastly, it should be mentioned that the optical scattering properties of tissue renders it opaque to light beyond a certain tissue depth. To circumvent this, groups have developed invasive endoscopy-based technologies to image deep brain structures (Barretto and Schnitzer, 2012) such as the hippocampus (Ziv et al., 2013), amongst others. While we recognize that investigations in such regions may lead to exciting insight into memory processing (Ziv et al., 2013) and beyond, a less invasive option that does not involve trauma to brain tissue should be considered. One option would be to develop an endoscopic imaging technology that can navigate through the vasculature and image deeper cortical structures.

Collectively, these technological advances combined with novel research ideas highlight the continuing importance of functional imaging research in the brain and for brain related diseases.
Bibliography


Kim, H., Li, Q., Hempstead, B.L., Madri, J.A., 2004. Paracrine and autocrine functions of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in brain-derived endothelial cells. J. Biol. Chem. 279, 33538-33546.


Curriculum Vitae

Darian Hadjiabadi  | dhadjia1@gmail.com| DOB: 06-23-1993  | Nationality: USA

Education

Ph.D.  Stanford University, Bioengineering – Palo Alto, California USA  Starting Fall 2017
M.S.E.  Johns Hopkins University, Biomedical Engineering – Baltimore MD, USA  Expected 2017
B.S.  Johns Hopkins University, Computer Science & Biomedical Engineering, Baltimore, MD USA  2015

Research and Professional Experience

Graduate Researcher (Masters)
Johns Hopkins University School of Medicine – Baltimore, MD USA  05/2015 – 08/2017
Supervisor: Arvind P. Pathak, Ph.D.
Thesis: Multi-scale functional imaging of cerebrovascular dynamics with application to brain tumors

Senior Undergraduate Researcher
Johns Hopkins University – Baltimore, MD USA  01/2015 – 05/2015
Supervisor: Emad M. Boctor, Ph.D.
Photoacoustic Neuroimaging

Systems Engineering Intern
Medtronic Neuromodulation – Fridley, MN USA  05/2014 – 08/2014
Supervisor: Steven J. Goetz

Undergraduate Researcher
Johns Hopkins University – Baltimore, MD USA  06/2012 – 08/2012
Supervisor: Michael I. Miller, Ph.D.
Computational analysis of LDDMM for brain mapping

Awards and Recognitions

Magna Cum Laude, ISMRM – 2017
ISMRM student travel grant – 2017

Peer-Reviewed Publications


Conferences


Teaching Experience

Teaching Assistant

Johns Hopkins University, Department of Biomedical Engineering – Baltimore, MD USA
Systems Bioengineering I – 580.421 – Fall 2015 – Supervising Instructor: Lawrence Schramm, Ph.D.
BME Pract & Innovation Lab – 585.525 – Summer 2017 – Supervising Instructor: Elizabeth A. Logsdon, Ph.D.

Lab Manager

Johns Hopkins University, Department of Biomedical Engineering – Baltimore, MD USA
BME Modeling & Design – 580.111 – Fall 2014 – Supervising Instructor: Eileen Haase, Ph.D.

Grader

Johns Hopkins University, Department of Biomedical Engineering – Baltimore, MD USA
System Bioengineering II – 580.422 – Spring 2017 – Supervising Instructor: Xiaoqin Wang, Ph.D.
Systems Bioengineering III – 580.429 – Fall 2016 – Supervising Instructor: Joel Bader, Ph.D.
Systems Bioengineering II – 580.422 – Spring 2016 – Supervising Instructor: Xiaoqin Wang, Ph.D.

Affiliations

Student Member, SPIE 09/2016 - Present
Trainee, ISMRM 09/2016 - Present
Alumni, Phi Delta Theta 05/2015 - Present
Brother, Phi Delta Theta 04/2013 – 05/2015
Secretary
09/2013 – 12/2013

Community Service

Relay for Life – Baltimore, MD USA 04/2017
Feed My Starving Children – Fridley, MN USA 05/2014 – 08/2014
President’s Day of Service – Baltimore, MD USA 09/2013
Indian River Memorial Hospital – Vero Beach, FL USA 01/2011 – 05/2011