Structured Illumination Diffuse Optical Tomography for Mouse Brain Imaging

Matthew Reisman
Washington University in St. Louis

Follow this and additional works at: https://openscholarship.wustl.edu/art_sci_etds

Part of the Optics Commons

Recommended Citation
https://openscholarship.wustl.edu/art_sci_etds/1204

This Dissertation is brought to you for free and open access by the Arts & Sciences at Washington University Open Scholarship. It has been accepted for inclusion in Arts & Sciences Electronic Theses and Dissertations by an authorized administrator of Washington University Open Scholarship. For more information, please contact digital@wumail.wustl.edu.
Structured Illumination Diffuse Optical Tomography for Mouse Brain Imaging
by
Matthew David Reisman

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

December 2017
St. Louis, Missouri
Table of Contents

List of Figures ......................................................................................................................... v
List of Tables ........................................................................................................................... vii
Acknowledgments ...................................................................................................................... viii
Abstract .......................................................................................................................................... xi
Chapter 1: Introduction ............................................................................................................... 1
  1.1 Functional and Anatomical Neuroimaging ................................................................. 1
  1.2 Optical Neuroimaging in Mice .................................................................................... 2
  1.3 Diffuse Optical Tomography ....................................................................................... 8
Chapter 2: Diffuse Optical Tomography in Mice Using Structured Illumination .............. 12
  2.1 Introduction .................................................................................................................. 12
  2.2 Methods ....................................................................................................................... 13
    2.2.1 Structured Illumination Diffuse Optical Tomography Imaging System .............. 13
    2.2.2 Light Modeling/DOT Forward and Inverse Problem ............................................. 15
    2.2.3 Animal Preparation and Imaging .......................................................................... 20
    2.2.4 Baseline Optical Property Estimation ................................................................. 21
    2.2.5 Data Analysis ......................................................................................................... 21
  2.3 Results ............................................................................................................................ 22
    2.3.1 Optimizing Measurements Using Effective Source-Detector Separation ............ 22
    2.3.2 Linearity of Diffuse Optical Tomography Measurements ...................................... 25
    2.3.3 In-Situ Estimation of Baseline Optical Properties ............................................... 25
    2.3.4 Measurement Removal .......................................................................................... 27
    2.3.5 Non-Invasive Evoked Responses In Vivo ............................................................... 28
    2.3.6 Resting State Functional Connectivity .................................................................... 31
  2.4 Discussion ......................................................................................................................... 33
    2.4.1 In Vivo Non-Invasive Activations ......................................................................... 34
    2.4.2 Context to Literature .............................................................................................. 35
    2.4.3 Limitations and Potential Improvements ............................................................... 36
  2.5 Summary/Conclusion ....................................................................................................... 38
Chapter 3: Whole-Brain DOT in Mice Using Multiple Camera Views .................................. 40
  3.1 Introduction ..................................................................................................................... 40
References/Bibliography/Works Cited ........................................................................ 88
Appendix: System Design/Construction Challenges ...................................................... 99
A.1 Introduction ............................................................................................................. 99
A.2 Structured Illumination DOT System Construction .................................................. 99
   A.2.1 Camera Selection ................................................................................................. 99
   A.2.2 LED and Wavelength Selection ......................................................................... 103
   A.2.3 Computer Selection ............................................................................................ 105
   A.2.4 Optics Selection ................................................................................................. 107
   A.2.5 Software ............................................................................................................. 108
A.3 Multi-view System Construction ............................................................................... 109
   A.3.1 Custom Light Engine .......................................................................................... 109
   A.3.2 Custom Projector ............................................................................................... 112
   A.3.3 System Speed ..................................................................................................... 113
   A.3.4 Computational Challenges ............................................................................... 115
List of Figures

Figure 1.1: Extinction coefficients of hemoglobin ................................................................. 3
Figure 1.2: Optical Intrinsic Signal (OIS) mouse imaging system ........................................ 4
Figure 1.3: Resting state functional connectivity in mice using OIS ...................................... 6
Figure 1.4: fNIRS and DOT measurements ........................................................................ 8
Figure 1.5: Previous DOT system overview ........................................................................ 9
Figure 2.1: Structured illumination DOT system .................................................................... 14
Figure 2.2: Simulation of DOT Green’s functions ................................................................. 16
Figure 2.3: Structured illumination DOT processing stream .................................................. 19
Figure 2.4: Measured light intensity vs. effective source-detector separation ...................... 23
Figure 2.5: Linearity of structured illumination DOT measurements ................................... 25
Figure 2.6: Correction scheme and light model quality assessment ....................................... 26
Figure 2.7: Measurement removal ......................................................................................... 28
Figure 2.8: Non-invasive imaging of evoked cortical responses in the mouse ....................... 29
Figure 2.9: Maps of evoked responses in individual mice ....................................................... 30
Figure 2.10: Evoked response time courses ........................................................................... 31
Figure 2.11: Resting state functional connectivity as a function of depth .............................. 32
Figure 3.1: Multi-view structured illumination DOT system .................................................. 42
Figure 3.2: Multi-view tomography system processing algorithms ....................................... 45
Figure 3.3: Surface profiling and 3D surface capture for projector-camera pairs ................... 46
Figure 3.4: Co-registration of multiple camera views ............................................................ 47
Figure 3.5: 3D mesh error estimation ..................................................................................... 49
Figure 3.6: Measurement 3D location assignment and optimization ..................................... 51
Figure 3.7: Forward model quality assessment ....................................................................... 53
Figure 3.8: Optimized optical properties for all six wavelengths ........................................... 54
Figure 3.9: Point perturbation simulation reconstructions ...................................................... 56
Figure 3.10: Multi-view tomography system sensitivity ......................................................... 57
Figure 3.11: Sensitivity and resolution of the multi-view structured illumination tomography system ................................................................. 59
Figure 4.1: Concurrent GCaMP fluorescence and OIS imaging system .............................. 67
Figure 4.2: Awake mouse imaging techniques ....................................................................... 69
Figure 4.3: GCaMP6 mouse model validation ................................................................. 72
Figure 4.4: Functional connectivity mapping between states in GCaMP6 mice ................. 73
Figure 4.5: ChR2-evoked hemodynamic responses depend on photostimulation parameters .... 75
Figure 5.1: Patterns of degeneration due to alcohol-induced apoptosis ................................ 81
Figure 5.2: MRI imaging volumes of mice after ethanol exposure during infancy .............. 82
Figure 5.3: Functional connectivity in adult mice after neonatal ethanol exposure .......... 83
Figure A.1: Quantum efficiency and SNR vs. wavelength .................................................. 101
Figure A.2: Detectivity of Andor Zyla camera .................................................................. 105
Figure A.3: System optical components arrangement .......................................................... 107
Figure A.4: Structured illumination DOT trigger sequences .............................................. 109
Figure A.5: Light engine ray diagram sketch .................................................................... 110
Figure A.6: Light engine lens selections and locations ......................................................... 111
Figure A.7: Custom projector ............................................................................................. 112
Figure A.8: Field of view vs. frame rate ............................................................................. 114
List of Tables

Table 2.1: Measurement removal percentages from an entire data set within each threshold ..... 28
Table 3.1: Surface profiling errors for various scan parameters ........................................ 62
Table 5.1: Brain volume % reduction vs. controls following ethanol exposure during infancy . 82
Table A.1: Light engine and custom projector power throughput ......................................... 113
Acknowledgments

When I accepted the offer from Washington University to attend graduate school here, I still was unsure if I was making the right decision. Not because I was reluctant, or because I had other strong opportunities I was passing up, but because of the uncertainty. Graduate school was this daunting mystery; dedicating the next 5+ years of my life to a to-be-determined field of scientific research, in a city I had never previously visited no less, was exceptionally overwhelming. Looking back, my worry was not entirely misplaced, as there were undeniably times of stress, doubt, and anxiety throughout, but I could not have been more wrong about whether or not this was the right decision. I would not change anything about my graduate experience if I could, and I owe a significant amount of appreciation to the many people along the way who were a part of my life and made this such an undeniably rewarding experience.

I am lucky to have had six committee members who each played a significant, and unique, role in my graduate career. First, I owe special recognition to Ralf Wessel, who more than any individual person contributed to my decision to attend Washington University. As a junior in college, when I first began exploring the idea of transitioning from “traditional” physics into more biomedical applications of physics, I looked into physicists who were studying the brain and sent emails to several of them asking what that transition might be like for me. While most professors sent me brief, impersonal messages (if anything at all), Ralf Wessel noticed my phone number in the email signature and called me, chatting with me for an hour about the field and encouraging me to read various recommended books and papers to learn more. I thought, if professors at Wash U treat random undergraduates from across the country with this level of personal support and attention, then that is a place I want to be. Sure enough, very soon after my
arrival, meeting Jim Miller proved that notion correct. He has been endlessly supportive, both on a personal and professional level, and I am incredibly lucky to have had him as a mentor.

If someone had asked me at the start of my graduate career what I wanted to for research and my eventual career, I would have said that I want to use my physics background to develop novel medical or imaging equipment. After my first conversation with Joe Culver, it was clear that his lab was more perfect for achieving that goal than I could have hoped, and I owe him a lot for giving me such an invaluable opportunity. Joe gave me the freedom and flexibility to design and construct an imaging system from the ground up, while providing necessary support throughout, and giving me the chance to routinely travel the world and share my exciting work with others, which has led to a number of indescribably rewarding relationships and experiences. I was lucky to join the lab at the same time that a post-doc, Adam Bauer, was beginning to transition to becoming a PI himself. As a result, Adam provided endless hands on mentorship as I was starting out in the lab, and I owe a lot of the skills I’ve gained in designing and troubleshooting optical systems to his guidance and support.

Another wonderful benefit of working with Joe Culver and Adam Bauer so closely has been their strong appeal to other talented scientists and engineers for collaboration. I was extremely lucky to be the graduate student paired up with Kevin Noguchi for one such collaboration, who has continuously provided me with exciting clinical applications of my work to pursue. Having his ongoing neuroscience and psychiatry expertise has regularly provided me with a reminder of the potential big picture value and application of my work, which at times throughout the grind of graduate school has been very important to keep in mind. I have similarly benefitted from the ongoing correspondence with Mark Anastasio, who, being an expert in some of the more challenge aspects of tomography and image reconstruction, has been a wonderful resource.
What has really made graduate school the most memorable and important period of my life, though, are the friendships I’ve built along the way. My labmates, Patrick Wright, Jonathon Bumstead, Karla Bergonzi, Zack Markow, Inema Orukari, and many other past and present members of the Culver lab, have not only made wonderful scientific contributions to my graduate career, but have provided such personal support and friendship over the last 4+ years that I am certain I would not have made it through graduate school without them.

The mentorship program in the physics department played a big role in my entire graduate career, and in particular, the mentors and older graduate students I met upon my initial visit, Ryan Murphy, Evan Groopman, Amber Groopman, Jeff Pobst, Tom Crockett, Anthony Kovacs, Mike Abercrombie, and many others, showed me how wonderful of a place this can be and gave me countless lifelong friends. Thankfully, my classmates and incoming students from subsequent years, Brendan Haas, Nick Weingartner, Nara Higano, Kim Sukhum, Mack Atkinson, Gus Medeiros, Kelsey Meinerz, Min Shinn, and honestly nearly an entire dissertation’s worth of additional people, continued to provide me with a wonderful and supportive group of friends.

But most of all, I owe more than I can say to Rachel Crouch, for her undeniable patience and support throughout all of graduate school. She committed to coming to St. Louis with me with even more mystery about what her life would be here than mine was, and she never complained once. She has stuck by my side through all the ups and downs of the previous 5+ years and I’ll never be able to show her enough gratitude for the support she has shown me.

Matthew Reisman

Washington University in St. Louis

December 2017
ABSTRACT OF THE DISSERTATION

Structured Illumination Diffuse Optical Tomography for Mouse Brain Imaging

by

Matthew David Reisman

Doctor of Philosophy in Physics

Washington University in St. Louis, 2017

Professor James G. Miller, Chair

Professor Joseph P. Culver, Co-Chair

As advances in functional magnetic resonance imaging (fMRI) have transformed the study of human brain function, they have also widened the divide between standard research techniques used in humans and those used in mice, where high quality images are difficult to obtain using fMRI given the small volume of the mouse brain. Optical imaging techniques have been developed to study mouse brain networks, which are highly valuable given the ability to study brain disease treatments or development in a controlled environment. A planar imaging technique known as optical intrinsic signal (OIS) imaging has been a powerful tool for capturing functional brain hemodynamics in rodents. Recent wide field-of-view implementations of OIS have provided efficient maps of functional connectivity from spontaneous brain activity in mice. However, OIS requires scalp retraction and is limited to imaging a 2-dimensional view of superficial cortical tissues. Diffuse optical tomography (DOT) is a non-invasive, volumetric neuroimaging technique that has been valuable for bedside imaging of patients in the clinic, but previous DOT systems for rodent neuroimaging have been limited by either sparse spatial sampling or by slow speed. My research has been to develop diffuse optical tomography for whole brain mouse neuroimaging by expanding previous techniques to achieve high spatial
sampling using multiple camera views for detection and high speed using structured illumination sources. I have shown the feasibility of this method to perform non-invasive functional neuroimaging in mice and its capabilities of imaging the entire volume of the brain. Additionally, the system has been built with a custom, flexible framework to accommodate the expansion to imaging multiple dynamic contrasts in the brain and populations that were previously difficult or impossible to image, such as infant mice and awake mice. I have contributed to preliminary feasibility studies of these more advanced techniques using OIS, which can now be carried out using the structured illumination diffuse optical tomography technique to perform longitudinal, non-invasive studies of the whole volume of the mouse brain.
Chapter 1: Introduction

1.1 Functional and Anatomical Neuroimaging

Rapid technological developments throughout the 1970s revolutionized neuroscience and hospital care by allowing physicians to non-invasively observe brain anatomy in their patients. These now-standard techniques for anatomical brain imaging, for example Computed Tomography (CT) [1], [2], Positron Emission Tomography (PET) [3], and Magnetic Resonance Imaging (MRI) [4], [5], are highly effective at providing clinical diagnoses of various neurological problems such as tumors, bleeding, and trauma, and are all still regularly used in the clinic to this day. Only in recent decades, however, has neuroimaging expanded into monitoring brain function as well as structure, accelerated by the discoveries that PET and MRI signals can be sensitive to changes in blood flow [6], [7]. Given the relationship between neuronal activity and hemodynamic activity in the brain (known as neurovascular coupling [8]), monitoring of blood flow using MRI serves as a good proxy for observation of brain activity. Although in the beginning functional MRI (fMRI) was typically used for task-based brain imaging [9], such as observing the increase in oxygenated hemoglobin in the visual cortex following a visual stimulus, the technique provided insight into many diseases that were previously limited to difficult psychological assessments, and has been the gold standard of functional neuroimaging ever since [10]. More recently, however, spontaneous fluctuations of blood oxygenation in the brain were found to persist even in the absence of tasks, and were found to be network dependent [11]. Specifically, spontaneous fluctuations in certain brain regions tend to occur in unison with others, implying some sort of functional connection between these regions, even in the absence of a direct anatomical connection. Studying this spontaneous activity within brain networks, now
referred to as resting state functional connectivity, has opened up functional brain imaging to some of the most sensitive and important populations that are incapable of performing the tasks required for traditional functional brain imaging, such as neonatal infants or those having suffered severe stroke or trauma [12], [13].

Resting state functional connectivity has also provided a new avenue for studying the mouse brain. The value of mice to biology and neuroscience is undeniable, as they can undergo highly controlled genetic and pharmacological manipulations to model various diseases and treatments. These important and clinically translatable studies can be greatly improved with functional neuroimaging, as longitudinal and minimally invasive studies are more accessible. Unfortunately, fMRI is difficult and expensive in mice, where very high magnetic field strengths are necessary to achieve sufficient signal-to-noise ratio (SNR) to detect resting state brain activity. However, while the small volume of the mouse brain is what limits the capabilities of fMRI (where signal is proportional to tissue volume), that exact characteristic works to the advantage of a different functional neuroimaging technique: optical imaging.

1.2 Optical Neuroimaging in Mice
In the same way that MRI signals are affected by changes in blood flow, light is preferentially absorbed and scattered by changes in blood oxygenation content as a function of its wavelength (Fig. 1.1) [14]. This can be easily demonstrated by shining a bright white light against one’s finger and seeing that only red light transmits through without being attenuated; shorter wavelengths are all completely absorbed within the first several millimeters of tissue. This phenomenon has become a regular part of everyday life in recent years, with smart watches detecting changes in reflected light intensity to measure heart rate or blood oxygenation. Recently, we developed an imaging system that uses this same technique to monitor brain
function throughout the mouse cortex by looking at the optical intrinsic signals (OIS) in the brain (Fig. 1.2A) [15].

Figure 1.1. Extinction coefficients of hemoglobin. Oxygenated hemoglobin (HbO₂, red) and deoxygenated hemoglobin (HbR, blue) have varying extinction coefficients as a function of wavelength over the visible and near-infrared parts of the spectrum. In general, longer wavelengths are less attenuated by hemoglobin, and therefore can penetrate deeper into biological tissue. Differences in reflected light intensity from multiple wavelengths can be used to determine the relative changes in HbO₂ and HbR.

The OIS imaging system is a planar imaging system, with a charge-coupled device (CCD) camera used to collect relative changes in reflected light intensity from light emitting diodes (LEDs) that sequentially and uniformly illuminate the exposed mouse skull (Fig. 1.2B). The system uses four different wavelengths, three with preferential sensitivity to deoxygenated hemoglobin (HbR) and one with preferential sensitivity to oxygenated hemoglobin (HbO₂) (Fig. 1.2C). The camera collects light from each wavelength of illumination separately, revealing stark differences in reflected light intensity in sequential frames (Fig. 1.2D-G). Shorter wavelengths, which have higher attenuation in tissue, show a greater contrast across the field-of-view (FOV), with blood vessels appearing more noticeable due to greater absorption of light in these regions.
Figure 1.2. Optical Intrinsic Signal (OIS) mouse imaging system. (A) The OIS system consists of a ring of LEDs of four different wavelengths which sequentially illuminate the mouse head and a CCD camera to collect relative changes in reflected light intensity. (B) A white light image generated from the OIS system, illustrating the camera’s point of view of the exposed mouse skull after scalp retraction and the 1cm x 1cm field of view. (C) The peak spectra locations of the four LEDs overlaid on the extinction coefficients of hemoglobin, showing blue preferentially sensitive to HbO₂ and yellow, orange, and red preferentially sensitive to HbR. (D-G) Four sequential images taken by the OIS system, one from each of the four illumination wavelengths. Given the 120Hz frame rate of the system, these four images span ~33ms, showing the significant differences in contrast between the four wavelengths.

Changes in reflected light intensity can be converted to changes in optical properties (specifically, changes in the absorption coefficient, as the scattering coefficient is relatively constant) using the Beer-Lambert Law:

\[ I(t) = I_0 e^{-\Delta \mu_a(t)L} \quad (1.1) \]

Here, \( I(t) \) is the time-dependent fluctuation of light intensity for a given wavelength and pixel, \( I_0 \) is the average (baseline) intensity in that pixel from that wavelength, \( \Delta \mu_a(t) \) is the time-dependent fluctuation in absorption coefficient at that pixel, and \( L \) is the assumed constant differential pathlength factor (DPF) [16] for that wavelength, which describes the average distance that the light travels through tissue. Calculating the DPF for each wavelength requires
some knowledge of the baseline (average) absorption and reduced scattering coefficients [17]. The baseline reduced scattering coefficient, $\mu'_s$, is reasonably constant in the visible spectrum in biological tissue [18] and therefore assumed to be 10cm$^{-1}$ for all wavelengths. The baseline absorption coefficient is calculated independently for each wavelength, as it depends on the spectra of the LEDs in use:

$$\mu_a = [HbT] \ast SO_2 \ast \left(\overline{HbO_2 \cdot LED}\right) + [HbT] \ast (1 - SO_2) \ast \left(\overline{HbR \cdot LED}\right)$$ (1.2)

Here, $\overline{HbO_2}$ and $\overline{HbR}$ are the extinction spectra from Fig. 1.1, $\overline{LED}$ is the normalized LED spectrum for the wavelength of interest, $HbT$ is the assumed baseline total hemoglobin concentration (76 μM), and $SO_2$ is the assumed baseline oxygen saturation percentage (0.71) [19]. With an estimate of baseline absorption coefficient for each wavelength, the DPF can be calculated (simplified from the definition by Arridge et al. [20]):

$$L = \frac{1 + 3\mu_a D}{2\mu'_s\sqrt{\mu_a D}}$$ (1.3)

Where $D$ is the diffusion coefficient, and is equal to $1/[3(\mu_a + \mu'_s)]$. Once the DPF has been calculated for each wavelength, the Beer-Lambert law can be rearranged to solve for the differential changes in absorption coefficient:

$$\Delta\mu_a(t) = \frac{-ln(I(t)/I_0)}{L}$$ (1.4)

Calculating this time-dependent change in absorption coefficient for multiple wavelengths gives rise to a linear system which can be solved to calculate the corresponding changes in HbO$_2$ and HbR:

$$\overline{\Delta\mu_a(t)} = \overline{E_{HbO_2}[\Delta HbO_2(t)]} + \overline{E_{HbR}[\Delta HbR(t)]}$$ (1.5)
The vector $\Delta \mu_a(t)$ is the time trace of changes in absorption coefficient for each wavelength in the system, and the vector $E$ for each of HbO$_2$ and HbR describes the extinction coefficient of each wavelength for those two contrasts. This extinction matrix is calculated by taking the dot product of each LED’s spectrum with the extinction coefficient spectra shown in Fig. 1.1. The solution of this linear system is the pair of independent time traces $\Delta HbO_2(t)$ and $\Delta HbR(t)$.

Once this linear system has been solved and the data have been converted to changes in HbO$_2$ and HbR concentration, the time traces are filtered to the canonical resting state functional connectivity band (~0.009-0.08Hz) [21], and maps of resting state functional connectivity are generated by comparing these filtered time traces of hemoglobin concentration fluctuation across different regions of the brain (Fig. 1.3A).

![Figure 1.3](image)

**Figure 1.3.** Resting state functional connectivity in mice using OIS. (A) Time traces of HbO$_2$ fluctuations with respect to baseline over a 5-minute period in a mouse from three different locations on the cortex (marked by corresponding colored dots in B). The green and blue traces are seen to fluctuate together strongly, while the red trace fluctuates roughly the opposite of them both. (B) A correlation map showing the Pearson-R correlation coefficient between every pixel’s time trace and that from the blue seed. For example, the correlated green and blue traces have a correlation coefficient of 0.92, while the anti-correlated blue and red traces have a correlation coefficient of -0.68. (C) Maps showing correlation coefficients between every pixel over the brain and 7 anatomical seeds of interest, shown in each map as a black dot.
By calculating the correlation coefficient between a particular brain region of interest (referred to as a seed) and every other pixel in the brain, maps of correlation coefficients can be calculated over the entire brain for that particular seed (Fig. 1.3B). Further, these seed-based functional connectivity maps can be calculated for a range of seed regions of anatomical interest, revealing the functional structure of the mouse brain (Fig. 1.3C). This can subsequently be used to study diseases and treatments by observing the disruption of these functional networks in the unhealthy mouse brain [22], [23].

While optical intrinsic signal imaging has proven very useful for monitoring brain function in mice and studying brain disease, it has a number of significant limitations. As previously discussed, OIS does not provide any information about baseline optical properties, and requires estimates or assumptions of them to ultimately reconstruct HbO$_2$ and HbR activity. Additionally, OIS imaging requires the minimally invasive procedure of scalp reflection prior to imaging. Given that a surgery and lengthy recovery are necessary, certain longitudinal studies, such as those in very young (infant) mice, are difficult to carry out using OIS. Furthermore, OIS provides no depth information because it is a planar imaging technique; we are limited to imaging the cortex, essentially looking at an average of all depths up to ~500µm beneath the brain’s surface. As a result, there are many inaccessible regions and higher order brain networks that cannot be observed using OIS.

The broad goal of my work is to expand an optical imaging technique that has recently shown promise in humans, Diffuse Optical Tomography, into mouse neuroimaging to address these weaknesses and provide new pathways for studying mouse brain function throughout the entire volume.
1.3 Diffuse Optical Tomography

Functional near-infrared spectroscopy (fNIRS) is a non-invasive optical imaging modality that, in contrast with planar imaging techniques, illuminates with a single source at a time [24]. By collecting light from multiple point detectors for each individual illumination, one can reconstruct changes within a volume based on how the different detectors collect light from the same illumination (Fig. 1.4A). The near-infrared light ($\lambda \sim 700$-900nm) in use can penetrate deep into tissue given its low attenuation (see Fig. 1.1), but traditional fNIRS techniques use sparse arrays of point sources and detectors. This confines the spatial resolution and causes non-uniform spatial sensitivity, which makes fNIRS a non-ideal candidate for rodent neuroimaging.

![Figure 1.4. fNIRS and DOT measurements.](image)

In recent years, however, Diffuse Optical Tomography (DOT) techniques have been developed that use the same framework as fNIRS, but incorporate much denser grids of sources and detectors to provide better spatial resolution and sensitivity throughout a large volume (Fig. 1.4B). Additionally, DOT typically uses computational modeling methods to model how light is expected to diffuse through the volume of interest. Discrepancies from this model are then used to attribute observed changes to specific locations throughout the volume (see Sec. 2.2.2), which provides much greater spatial accuracy than simply correlating location with source-detector separation as is typically done in fNIRS.
Consequently, DOT is a much better candidate for whole-brain, non-invasive functional neuroimaging than fNIRS, as several early systems demonstrated. A fiber-based DOT system, consisting of ~10 point sources and ~10 point detectors, has shown the ability to image the rat cortex non-invasively with sufficient speed for monitoring hemodynamics (Fig. 1.5A), but this fiber array is too sparse to provide the whole-brain sensitivity that we hope to achieve with a mouse DOT system. The sparseness limitation has been addressed with a high-density fiber-based DOT (HD-DOT) system for human neuroimaging. This technique implements finite element modeling of light transport in tissue, which helps to provide volumetric reconstructions of brain activity with good resolution and sensitivity, but the HD-DOT fiber array cannot easily be scaled down to the size of the mouse head (Fig. 1.5B).

![Figure 1.5. Previous DOT system overview.](image)

(A) A fiber-based DOT system for rodent neuroimaging [25], with too sparse of spatial sampling for the desired mouse neuroimaging applications. (B) A fiber-based high-density DOT system for human neuroimaging [26], which cannot be easily scaled to the size of the mouse head while preserving dense spatial sampling. (C) A CCD and laser diode-based DOT system for molecular and fluorescence imaging in rodents [27], which is too slow to monitor hemodynamics in the mouse brain. (D) Structured illumination using a spatial light modulator [28], which has been applied to numerous optical imaging techniques, such as optical tomography and microscopy. We aim to combine the relative strengths of each of these (green text) into a structured illumination DOT system for mouse neuroimaging.

The measurement density problem has successfully been solved for rodent DOT by, instead of using fiber-based sources and detectors, raster scanning a laser diode over the volume of interest and using a CCD camera for detection (CCD-DOT, Fig. 1.5C). In this case, each pixel of the
CCD sensor acts as an independent detector. While this has proven useful for molecular fluorescence imaging, which is a valuable technique for imaging different contrasts than just hemoglobin (see Ch. 4), CCD-DOT is too slow for imaging hemodynamics in the brain.

Most recently, speed issues have been addressed in similar systems by using a spatial light modulator to convert uniform planar light into spatially varying 2D illumination, referred to as structured illumination source patterns. Structured illumination patterns provide rapid whole sample illumination instead of only illuminating one point at a time (Fig. 1.5D). Traditional uses of this technique require sine wave spatial patterns, however, and are therefore not applicable to the arbitrary illuminations and geometries that would be desired for a mouse DOT system [29].

We can address each of the issues that has limited previous DOT techniques from performing whole brain mouse neuroimaging by combining their respective strengths into what will be the primary focus of the remainder of this work: Structured Illumination Diffuse Optical Tomography (SI-DOT). This technique combines the speed of structured illumination sources with the high-density detectors of the camera-based CCD-DOT and the finite element modeling of the fiber-based human HD-DOT into a system capable of fast mouse neuroimaging with good resolution throughout the cortex and good sensitivity to subcortical brain structures. Chapter 2 of this work discusses the development of the preliminary SI-DOT system, the theory and analysis techniques used to optimize the system, and a feasibility study showing its ability to perform non-invasive functional neuroimaging in mice. Chapter 3 discusses the expansion of the SI-DOT system into its full form: a “multi-view” tomography system with two separate projectors for multiple illumination angles and three cameras for multiple detection angles. Chapter 3 also focuses on the addition of an in situ surface profiling technique to greatly increase the accuracy
of the forward modeling of light transport in tissue and subsequently the system sensitivity and resolution throughout the volume of the mouse brain.

Chapters 4 and 5 discuss preliminary work that has been carried out on problems that will eventually be addressed more thoroughly with the full, multi-view tomographic imaging system. Chapter 4 focuses on a system developed to perform awake mouse imaging, and highlights some results showing the ability of this system to image mice while awake and without brain function potentially confounded by anesthesia. Additionally, this chapter summarizes this system’s ability to observe higher order networks using contrasts besides just hemoglobin, via techniques such as optogenetics and calcium fluorescence imaging, which can ultimately be applied to DOT as well.

Chapter 5 focuses on a preliminary study of a mouse model of fetal alcohol syndrome (FAS). Further motivating the removal of anesthesia from the imaging of mice, many common anesthetics or sedatives given to infants, which fall in the same class of drugs as ethanol, produce excessive spontaneous cell death in the brain and likely contribute to many of the functional deficits associated with FAS. This work aims to determine the ability of optical imaging to detect these deficits, with eventual work expanding to more commonly used drugs besides just ethanol. Establishing which drugs affect brain development and functional connectivity would have wide-ranging clinical applications, and the flexible non-invasive mouse DOT imaging system will be the ideal means to carry out such a study.
Chapter 2: Diffuse Optical Tomography in Mice Using Structured Illumination

2.1 Introduction
As advances in functional magnetic resonance imaging (fMRI) have transformed the study of human brain function, they have also widened the divide between standard research techniques used in humans and those used in mouse models. Although both task-based evoked responses [30], [31] and resting state networks [32], [33] have recently been observed in mice using fMRI, high signal-to-noise ratio (SNR) and resolution remain challenging to achieve in the small volume of the mouse brain, and the logistics of MRI hinder widespread application to high-throughput mouse studies. A need exists for a fast benchtop modality for studying brain networks in mice. Optical imaging techniques, such as optical intrinsic signal imaging [15] (OIS, see Sec. 1.2), have been developed and widely applied to task-based evoked responses [34], [35]. Most recently, OIS has been applied with a wide field-of-view (FOV) to monitor functional connectivity in cases of disease [22], [23] and development [36] in the mouse brain. However, traditional OIS methods are limited to planar imaging, providing only a two-dimensional view of cortical activity. Further, planar imaging requires, at the least, the minimally invasive procedure of scalp reflection, making longitudinal imaging difficult or even impossible in some populations, such as infant mice.

In contrast, Diffuse Optical Tomography (DOT) provides non-invasive volumetric imaging at depths extending to multiple centimeters, which in principle solves some of the limitations of planar imaging. In addition to DOT instrumentation, algorithms have been developed for handling arbitrary tissue geometries that can be matched to anatomy using numerical finite element modeling of light transport [37]. While most papers have focused on humans [26], [38],
there have been some reports of the application of DOT to rodents [25], [27]. However, thus far most animal DOT systems have either been fiber-based, which are limited by sparse spatial sampling [25], or CCD-based, which are limited by slow frame rates (<0.1 Hz) that preclude imaging functional brain hemodynamics [27].

Here, we present an imaging system that combines structured illumination (SI) with traditional DOT techniques (SI-DOT) to image a wide FOV (>1 cm x 1 cm) at high speed (>2 Hz). Successful implementation of SI-DOT for mouse functional neuroimaging requires optimizing for structured pattern sequences that preferentially select deeper tissue. We introduce an analysis of the SNR of these patterns that evaluates the average light intensity as a function of an effective distance. This provides a light intensity versus distance analysis analogous to methods used in traditional fiber-based DOT. Following optimization, we validate SI-DOT for non-invasive imaging in mice by observing, through the intact scalp, cortical responses to peripheral stimulation.

2.2 Methods

2.2.1 Structured Illumination Diffuse Optical Tomography Imaging System

The goal of the structured illumination diffuse optical tomography instrument is to provide non-invasive functional neuroimaging of cortical hemodynamics (through both the scalp and skull) at a speed >2 Hz with a FOV >1 cm² to cover the dorsal convexity of the mouse brain. The system leverages fast, low noise detection provided by a single scientific complementary metal-oxide-semiconductor (sCMOS) camera (Zyla 5.5, Andor Technology Ltd., South Windsor CT, USA). For multi-wavelength structured illumination, we used a single projector (Lightcrafter 4500, Texas Instruments, Dallas TX, USA). Within the projector, multi-colored LEDs were reflected off a 912 x 1140 digital micromirror device (DMD) array to display arbitrarily complex two-
dimensional illumination patterns. The sizes of the DMD chip, the mouse head, and the sCMOS sensor were all similar, which allowed for symmetric imaging optics.

Figure 2.1. Structured illumination DOT system. (A) System schematic showing the relative positions and orientations of the DMD source projector and sCMOS camera. Internal LEDs and optics illuminate the DMD with the desired wavelength, and illumination patterns are stored in the on-board projector memory and triggered consecutively to illuminate the head with the desired spatial frequencies. (B) Six example structured light patterns illuminating the intact mouse scalp, as collected by the sCMOS. (C) The planar-frame illumination of a mouse head as measured by the sCMOS camera, showing the positions of ~1,000 detectors (blue) over the intact scalp, after off-camera binning. A hand-drawn brain mask (yellow) removes measurements that lie outside of the exposed scalp for each mouse.

In particular, 85mm f/1.4 lenses were used to maximize the FOV while allowing sufficient working distance so that the projector could illuminate the mouse head from above (Fig. 2.1A).

To prevent specular reflection off the scalp from saturating the sensor, a polarizer (B+W 72mm XS-Pro Kaesemann, Schneider Optics, Van Nuys CA, USA) was placed on the projector with its polarization axis 90° relative to a second polarizer in front of the camera lens. A sequence of pre-defined illumination patterns was created in Matlab (Mathworks, Natick, MA) and uploaded onto the projector’s on-board memory. Individual patterns were triggered one at a time and synchronized with each camera frame (Fig. 2.1B). Several illumination sequences were explored, typically containing 40 different structured patterns. Each pattern ranged in spatial frequency from 0.08 to 0.4 mm\(^{-1}\) with two phases (180-degree phase shifts) and two orientations included per frequency. Images were collected at a camera frame rate of 80 Hz, providing a full DOT frame rate of 2Hz. Each detection frame spanned a 12mm x 12mm FOV using the central 512 x
512 pixels of the sensor. The data were binned to 32 x 32 pixels prior to reconstruction to improve SNR, yielding a pixel size of ~400um. The binned data had a dynamic range of $10^4$, with typical maximum values of ~$10^7$ counts and background standard deviations of approximately $10^3$ counts. The 40 illumination patterns combined with ~1,000 detectors over the scalp provided ~40,000 total measurements (Fig. 2.1C).

2.2.2 Light Modeling/DOT Forward and Inverse Problem

Diffuse Optical Tomography Image Reconstruction

The diffusion of light through biological tissue can be described by the time-independent diffusion equation, an approximation of the radiative transport equation [39]:

$$D \nabla^2 \Phi(\vec{r}) - \nu \mu_a(\vec{r}) \Phi(\vec{r}) = -\nu S(\vec{r})$$

(2.1)

Here, $\nu$ is the speed of light in the medium, $\Phi$ is the photon fluence (light intensity per unit area), $S$ is the source distribution, and the diffusion coefficient $D = \nu/[3(\mu_a + \mu_s')]$, where $\mu_a$ and $\mu_s'$ are the absorption and reduced scattering coefficients, respectively. The scattered field (fluence) can be solved for using the Rytov approximation [40]:

$$\Phi(\vec{r}) = \Phi_0(\vec{r}) e^{\Phi_1(\vec{r})}$$

(2.2)

Here, the small perturbations in the light fluence, $\Phi_1$, are much smaller than the baseline, unperturbed fluence $\Phi_0$. Given that the data are ratiometric, we can solve for relative changes in absorption at each location within the volume by solving the linear system:

$$y = Ax$$

(2.3)

where $y$ is the solution of Eq. 2.2 for the perturbed fluence, a vector of differential light measurements for each source-detector pair:
\[ y = \Phi_1 = \ln\left(\frac{\Phi}{\Phi_0}\right) \]  

(2.4)

\( A \) is the sensitivity matrix, describing the sensitivity of each source-detector pair (i) in the system at each voxel location (j, with voxel volume \( h^3 \)):

\[ A_{i,j} = -\frac{v h^3}{D} \frac{G(\vec{r}_{s,i}, \vec{r}_j) G(\vec{r}_j, \vec{r}_{d,i})}{G(\vec{r}_{s,i}, \vec{r}_{d,i})} \]  

(2.5)

And \( x = \Delta \mu_a(\vec{r}_j) \), the change in absorption coefficient at each voxel location. The sensitivity matrix consists of three Green’s functions: A source Green’s function (Gs) describing the fluence at each voxel location due to a point source illumination; a detector Green’s function (Gd) describing the light propagation from each voxel location to a given point detector;

**Figure 2.2.** Simulation of DOT Green’s functions. (A) A coronal slice of a cylindrical model of a mouse head, showing simulated point source illumination and simulated point detector (i.e. a camera pixel) locations. (B) The source Green’s function (Gs), showing the exponentially decaying fluence throughout the slice due to the point illumination. (C) The detector Green’s function (Gd), mathematically identical to Gs but at the location of the detector instead. (D) The normalized system sensitivity (Gsd) at every voxel location throughout the slice for this example source-detector pair.
and a normalization Green’s function (Gsd) describing the fluence at the detector’s location due to illumination from the point source (Fig. 2.2).

The sensitivity matrix is directly inverted via Tikhonov regularization following methods used commonly for human DOT data [26], [37], [41]. Briefly, because $A$ is not square, a pseudoinverse is calculated instead:

$$A^\# = A^T [AA^T + \lambda \cdot \max\{\text{diag}(S)\} I]^{-1}$$  \hspace{1cm} (2.6)

$A^T$ is the transpose of the sensitivity matrix $A$, $I$ is the identity matrix, $S$ is the diagonal matrix of singular values of $A$, and $\lambda$ is the threshold for masking singular values with respect to the maximum singular value, below which corresponding modes are not included in the reconstruction. Additionally, to prevent the exponential decay of light with depth in the volume from reconstructing data too superficially, a second regularization can be used to spatially normalize the sensitivity matrix using a matrix $L$ such that

$$\text{diag}(L) = \sqrt{\text{diag}(A^T A)} + \beta \cdot \max\{\text{diag}(A^T A)\}$$  \hspace{1cm} (2.7)

Where $\beta$ is chosen to normalize voxels with low sensitivity and prevent inaccurate depth localization deep in the reconstruction volume. Now the spatially normalized sensitivity matrix, $\tilde{A} = L^{-1} A$, can be inverted as in Eq. 2.6 and multiplied by the log-ratio of the light measurements (Eq. 2.4) to produce a volumetric image sequence of absorption perturbations over time. After reconstructing changes in absorption coefficient for multiple wavelengths, these data are converted to changes in chromophore concentration using the extinction coefficients of each hemoglobin species at each of the wavelengths in use, as discussed in Sec. 1.2 [14].
Structured Illumination DOT Forward Modeling
To model the diffusion of light in tissue from structured illumination sources, first a finite
element mesh of an optically homogeneous tissue slab (right rectangular prism geometry) is
generated using NIRFAST, an open-source software package for modeling light diffusion [37],
[42]. For a set of chosen baseline optical properties, we solve for the point light propagation from
each mesh surface normal using the linearized Rytov forward model outlined in the previous
section. The finite element mesh is then resampled to the desired voxelated space. The source
patterns are estimated in situ to infer which locations on the tissue surface were illuminated by
each pattern. Illuminated points are defined to be those greater than 50% of the maximum value
in each frame. To account for noisy edges, for example due to mouse head curvature or skin
pigment variation, light-dark line boundaries in each pattern are defined to be where <50% of the
points in a line profile are illuminated. The corresponding point Green’s functions are summed to
create the fluence distribution for each structured illumination pattern. This is valid as only first
order interactions between the electromagnetic field and the tissue need to be considered; any
non-linear interactions, such as two-photon absorption, are negligible. This assumption is
commonly invoked in DOT to model realistic shapes (i.e., exponential decay as a function of
depth and finite beam areas) for point illumination [43]. Additionally, linear approximations to
the diffusion equation are used extensively in the DOT literature, namely the Rytov [44], Born
[45], and normalized Born approximations [46]. These linear assumptions are particularly well-
suited for detecting brain activity where the perturbations in absorption are small. To confirm
that these linear assumptions are valid for structured illumination DOT measurements, we
collected data with both extended illumination structures and as a series of separate images with
point illuminations. Both approaches agree within experimental SNR (see Sec. 2.3.2).
The predicted fluence at the tissue surface for each source-detector pair (Gsd, the unperturbed fluence) is converted into predicted detector counts for the corresponding measurement and compared to the raw data. Inaccuracies are addressed by iterating through the forward model’s generation of Green’s functions using different baseline optical properties until the fit between raw data and modeled data is optimized (see Sec. 2.3.3). The products of the source and detector Green’s function values at each voxel are normalized by Gsd for the corresponding source-detector pair and scaled by the voxel volume.

This quantity for each source-detector pair forms a row of the sensitivity matrix, whose columns correspond to voxels. A regularized pseudo-inverse of the sensitivity matrix is then calculated using spatially-variant Tikhonov regularization (with $\lambda=0.01$ and $\beta=1$, following the notation of Dehghani et al. [47], see eqs. 2.6 and 2.7), and this regularized pseudo-inverse is applied to the
experimental measurements to reconstruct images (i.e., solve the inverse problem). This entire process is repeated to construct a separate sensitivity matrix and independently reconstruct images for each illumination wavelength. Light modeling and data analysis are conducted along parallel processing pipelines, adapted from our previous methods for High Density DOT (Fig. 2.3) [26].

2.2.3 Animal Preparation and Imaging
All animal studies were approved by the Washington University School of Medicine Animal Studies Committee (protocol #20160217) under guidelines and regulations consistent with the Guide for the Care and Use of Laboratory Animals, Public Health Service Policy on Humane Care and Use of Laboratory Animals, the Animal Welfare Act and Animal Welfare Regulations, and ARRIVE guidelines. Male C57/BL6 mice (n=5, 5-7 weeks, 26-32g, Jackson Laboratories, Bar Harbor, ME, USA) were used for imaging. At least one day before imaging, mice were anesthetized with 2% isoflurane for hair removal using Veet. For imaging, mice were anesthetized with a ketamine-xylazine mixture (86.9 mg/kg ketamine, 13.4 mg/kg xylazine) at 5μL/g with body temperature maintained at 37 degrees C using an electric heating pad (mTCII, Cell Microcontrols, Norfolk VA, USA). Mice were secured using a Dazai imaging sled (Dazai Research Instruments, Toronto, Canada), with Velcro straps behind the ears and over the nose to prevent motion artifacts due to breathing. Each mouse was imaged for 35 minutes. Forepaw electrical stimulation was performed using A-M Systems Model 2100 Isolated Pulse Stimulator (A-M Systems, Sequim WA, USA), with trigger sequences syncing stimulation, camera frames, and structured light patterns created in Matlab (Mathworks, Natick MA, USA) and generated using a National Instruments Analog Output DAQ (National Instruments, Austin TX USA).
2.2.4 Baseline Optical Property Estimation
Optimal baseline optical properties are estimated by first averaging the raw data across all time points for each illumination pattern. Each frame has the background frame subtracted and is normalized by the planar frame. The average signal vs. effective source-detector separation (see Sec. 2.3.1) is calculated for each wavelength at each unique distance. Gsd is calculated for a range of values of μa (see Sec. 2.3.3), which provides a predicted fit. The optimal μa is chosen to be the value that minimizes chi-squared error between fit lines of corrected data and predicted data (see Fig. 2.6B).

2.2.5 Data Analysis
Differential raw data are collected at 512x512 pixels covering a 12mm x 12mm FOV and binned down to 32x32 pixels off camera for increased SNR and a more computationally efficient inverse problem. To account for systematic drift over the course of an imaging session, binned data are temporally de-trended by subtracting the fit of a 5th order polynomial to each pixel’s time trace. The shape of each individual structured light pattern is then empirically determined to generate the source Green’s functions. The signal vs. effective source-detector separation is calculated for each measurement, and noisy measurements are identified and removed by setting a threshold at 20x the typical background standard deviation (see Fig. 2.4D). The de-noised data are transformed by normalizing each measurement’s time trace by its mean and taking the logarithm to permit reconstruction of images. The average time trace of superficial measurements (those with effective source-detector separation less than 0.5mm, which mostly probe shallow, non-cortical depths) is then regressed from the data set to remove systemic scalp signals. Analogous superficial regression procedures have been employed for similar purposes in previous DOT work [26], [38], [41]. After reconstruction, data are temporally band-pass filtered between 0.009 and 0.25 Hz and spatially smoothed by convolution with a 3D Gaussian kernel. Data from both
wavelengths are reconstructed, with spectroscopic inversion using the LED spectra and known extinction coefficients from Prahl, adapted from our previous methods ([14], [15]).

Each individual five-minute run is block averaged. To include a run in group averages, data are required to have a visually identifiable evoked response, peaking temporally during or very near the stimulus period and with magnitude at least twice as large as peak fluctuations outside the activation region. All runs that fit these criteria are averaged to generate temporal and spatial maps of oxygenated hemoglobin (HbO₂), deoxygenated hemoglobin (HbR), and total hemoglobin (HbT) concentration dynamics for each mouse. Time traces are plotted by determining the time and depth of the peak activation, taking the mean of that layer and the layers immediately above and below, and then averaging the time traces from all pixels that reach magnitude greater than 50% of the peak activation in the maximum frame. Group averaged maps are generated by 2D translating the maps of each individual mouse in parallel axial planes such that the peak activations all align with their collective center of mass.

2.3 Results

2.3.1 Optimizing Measurements Using Effective Source-Detector Separation

A common technique for assessing measurement quality in diffuse optical tomography is to examine the measured light-level intensity as a function of source-detector separation. For structured illumination DOT, the source-detector separation is not as conceptually obvious as the Euclidean distance between the point-like sources and detectors in fiber-based DOT systems. However, a similar analysis would still be useful for evaluating measurement noise. Here we defined an effective source-detector separation (Eq. 2.8) between each dark detector pixel and the source pattern for every measurement (Fig. 2.4A, 2.4B). If one assumes an exponential fall-off of light fluence as a function of distance (e.g. following the Beer-Lambert law) and an
effective attenuation coefficient of 1mm\(^{-1}\) (a sensible value for the wavelengths in use), then a reasonable definition for an effective source-detector separation between a detector (pixel) and an illumination pattern is the exponentially weighted average of distances to each illuminated pixel. Briefly, for a dark detector pixel \(i\), source pixel \(j\), and source pattern \(k\), the effective distance is defined as:

\[
EffectiveDistance(i, k) = \frac{\sum_{j \in \Omega_k} \left| r_i - r_j \right| e^{-\mu_{eff} \left| r_i - r_j \right|}}{\sum_{j \in \Omega_k} e^{-\mu_{eff} \left| r_i - r_j \right|}}
\]  

(2.8)

where \(r_i\) and \(r_j\) are the locations of dark detector pixel \(i\) and source pixel \(j\). \(\mu_{eff}\) is defined to be 1mm\(^{-1}\), and \(\Omega_k\) is the set of bright pixels contained in source pattern \(k\). Each pair of vertical bars denotes the 2-norm of the enclosed expression, so \(|r_i - r_j|\) is the ordinary Euclidian distance between \(r_i\) and \(r_j\).

**Figure 2.4.** Measured light intensity vs. effective source-detector separation. (A) Empirically determined edges of illuminated regions for one example pattern. (B) The effective source-detector separation is calculated for each detector in the dark region of each source pattern, shown here for one example pattern. (C) Total number of measurements as a function of distance, binned into 0.25mm groups, for an illumination sequence consisting of 10 spatial frequencies and 1 color (orange) and a sequence of 5 frequencies and 2 colors (blue). (D) Signal intensity vs effective source-detector separation for each individual measurement, with averages at each unique distance emphasized and linearly fit. Data below the noise threshold (20x background standard deviation) were cropped prior to reconstruction.
In principle, the set of possible patterns and pattern sequences is quite extensive as the problem expands combinatorially. We chose to evaluate binary square wave patterns that provide greater dynamic range and can be triggered faster using our projector in comparison to sine waves, which are typically used in modulated light imaging [29] or structured illumination microscopy [48]. We first evaluated ten spatial frequencies with two phases (180° offsets) and two orientations (vertical and horizontal) each. For this pattern set, we found that over 75% of the measurements had an effective source-detector separation of 1mm or less (Fig. 2.4C, orange). By sacrificing the five highest spatial frequencies, a dense set of measurements with short separation distances is preserved while the relative sampling at larger distances is increased, improving the sensitivity to deeper tissue (Fig. 2.4C, blue). With fewer spatial frequencies, the pattern sequence was expanded to include two wavelengths for spectroscopic reconstructions of multiple chromophores (i.e. HbO₂, HbR and HbT) while keeping the full frame rate at 2Hz.

The average signal among all measurements at each unique effective source-detector separation showed an expected log-linear fall-off. In addition to guiding optimization of the illumination patterns, this technique helped us quantitatively identify noisy measurements. For example, the signals from green (λ = 523nm) and red (λ = 625 nm) illumination patterns began to approach the noise floor of the camera at effective source-detector separations greater than 3mm and 4.5mm, respectively (Fig. 2.4D). We empirically determined a threshold for cropping bad measurements by examining the standard deviation of light intensity in the background frames. Measurements greater than 20 times this value (e.g., > ~10^4.5 electrons for a representative measurement) were used for all reconstructions. This noise threshold removed approximately 50% of all measurements, the majority of which fell outside of the visually identified brain region (see Sec. 2.3.4).
2.3.2 Linearity of Diffuse Optical Tomography Measurements
In order to verify that our structured illumination measurements are linear, as has been shown for traditional point-based DOT [44], [45], we performed measurements of point illuminations in a phantom. For a given pattern illumination (Figure 2.5A), we generated a grid of equal sized point illuminations that scan the same area with no overlap between them (Figure 2.5B). Given that each point illumination frame is collected using the same exposure time as a single frame of the full pattern, we can compare a sum of the point illumination frames with full structured pattern frame (Figure 2.5C). Comparing the summed point illumination and structured pattern frames shows that the percent error between the two is generally less than 10% (Figure 2.5D), implying that the sum of point illuminations is linear. Thus, our forward model, which treats the structured light Green’s functions as sums of point Green’s functions, is appropriate.

![Figure 2.5. Linearity of structured illumination DOT measurements.](image)

**Figure 2.5.** Linearity of structured illumination DOT measurements. (A) An 8x15 grid of square points covering the same area as a structured pattern of interest is scanned on a phantom. One example point illumination frame is shown, before binning or background subtraction, with the grid illustrating the locations of the remaining points. (B) The sum of the full grid of illumination points, after binning down to 32x32 pixels, subtracting a background frame from each image and averaging over 19 cycles. (C) Single frame of the equivalent structured illumination pattern, after binning down to 32x32 pixels, background subtraction and averaging over 19 cycles. (D) The ratio of the SI frame and the summed point frame, showing uniform agreement between the two, within the expected variance due to greater noise in the point-summed frame.

2.3.3 In-Situ Estimation of Baseline Optical Properties
The sensitivity matrix used for DOT image reconstruction requires an estimate of the baseline tissue optical properties. Often, DOT algorithms use assumed attenuation coefficients from extant literature rather than in situ measurements. The data acquired in structured illumination DOT have the potential to permit in situ estimation of baseline optical properties by comparing
the raw collected data to a light model. While the continuous wave data used here cannot separate absorption and scattering, the data can be used to fit for the effective attenuation coefficient, from which empirical estimates of the in situ absorption coefficients can be deduced given an assumed reduced scattering coefficient of 10 cm\(^{-1}\). Non-uniformities in illumination and skin pigment, however, can distort the interpretation of raw structured illumination DOT measurements. The spatial inhomogeneities in the reflected light intensity of each frame were corrected by subtracting a dark frame from each raw image and normalized by a frame of uniform illumination. Reflected light intensity in each corrected frame was then compared to a light model (Fig. 2.6A).

**Figure 2.6.** Correction scheme and light model quality assessment. (A) Raw data, corrected data, and predicted data for both green and red illumination. (B) The signal vs. effective source-detector separation is calculated for the raw data, corrected data, and predicted data for a range of different absorption coefficients. Optimal \(\mu_A\) values are shown as minima in the chi-squared plots between the corrected data and predicted data and were empirically determined to be 3.33 cm\(^{-1}\) (top) and 0.34 cm\(^{-1}\) (bottom) for 523nm and 625nm wavelengths, respectively. (C) The signal vs. effective source-detector separation for the corrected data fit with optimal \(\mu_A\) values for red and green wavelengths.

For in situ estimation of optical properties, we compared the light fluence fall-off as a function of effective distance for both the structured illumination DOT measurements and the model predicted data, given by the source-detector Green’s functions (see Sec. 2.2.2). The chi-squared error between data and model was a continuous function of \(\mu_A\), with best fit values of \(\mu_A\) at 3.33
cm\(^{-1}\) and 0.34 cm\(^{-1}\) for 523nm and 625nm wavelengths, respectively (Fig. 2.6B). Fits using the optimal \(\mu_a\) values showed excellent agreement to the data (Fig. 2.6C).

These fit lines also provided a means for refining our initial data quality assessment. Measurements with >40% disagreement with the optimal predicted light fall-off were cropped from the dataset prior to reconstruction. This model-deviation threshold typically removed less than 10% of the total measurements (see Sec. 2.3.4). Finally, superficial measurements (defined to be those with an effective source-detector separation less than 0.5mm) were averaged and regressed from all remaining measurements prior to reconstruction. This is analogous to superficial signal regression performed in human brain DOT and reduces the contribution of hemodynamics outside the brain (e.g., in scalp) to the measured cortical signals [26], [38], [41].

2.3.4 Measurement Removal
A series of masks are applied to reduce the measurement set to only include those of sufficient quality in the reconstruction. Upon data collection, 40 source patterns and the 32x32 grid of binned detectors combine to provide 40,960 measurements. A visually identified and manually constructed brain mask excludes measurements in the FOV that do not lie over the scalp (Fig. 2.7B). This mask typically identifies 40-45% of the total measurements. The noise threshold discussed in Sec. 2.3.1 typically removes 50% of the measurements, those which fall within 20x the value of the standard deviation of the background. Nearly all of these measurements overlap those removed by the brain mask (Fig. 2.7C). A model mask determines which measurements deviate from expectation, based on the optimized model (as discussed in Sec. 2.3.3). This mask typically removes less than 10% of the measurements with green illumination, and less than 5% of the measurements with red illumination (Fig. 2.7D). Finally, the overlap of these three masks is the complete set of measurements to be used in reconstructions (Fig. 2.7E).
Figure 2.7. Measurement removal. (A) Raw corrected data for four different green illumination patterns, a high and low spatial frequency for each orientation. (B) The brain mask removes measurements from pixels that do not approximately align over the scalp and brain. This mask is identical for all illumination patterns for a given mouse. (C) The noise mask shows which measurements were below the noise threshold for the example pattern. (D) The model mask shows which measurements differed by more than 50% from the model’s prediction. (E) Only measurements surviving all 3 mask procedures are kept. This procedure is repeated for all illumination patterns and wavelengths to determine the full structured illumination DOT measurement set used for reconstruction.

Table 2.1 Measurement removal percentages from an entire data set within each threshold.

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>% of Total Measurements within noise threshold (Fig 2.7C)</th>
<th>% of Total Measurements within model threshold (Fig 2.7D)</th>
<th>% of Total Measurements in reconstruction (overlap)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red (623 nm)</td>
<td>51.9%</td>
<td>97.1%</td>
<td>49.0%</td>
</tr>
<tr>
<td>Green (530 nm)</td>
<td>48.3%</td>
<td>91.5%</td>
<td>41.5%</td>
</tr>
</tbody>
</table>

2.3.5 Non-Invasive Evoked Responses in Vivo
To validate structured illumination DOT for non-invasive functional mouse neuroimaging, we imaged anesthetized mice during peripheral stimulation of the left forepaw in a block design. Each block contained 10 seconds of 300μs, 0.5mA pulses delivered at 3 Hz followed by 50
seconds of rest. A two-wavelength structured illumination sequence allowed reconstruction of voxelwise changes in HbO₂, HbR, and HbT. The reconstruction geometry (12mm x 12mm x 2mm, with voxel dimensions of 32x32x20, width x length x depth) covered a FOV consisting of scalp, skull, and upper cortical layers (Fig. 2.8A). Five mice were imaged for 35 minutes each. Of this dataset, 120 minutes of data passed measurement quality thresholds and were used for block averaging. Total hemoglobin block averaged images show focal activity in the contralateral (right) forepaw region (Fig. 2.8B).

Figure 2.8. Non-invasive imaging of evoked cortical responses in the mouse. (A) Dotted lines show the FOVs for axial and coronal slices. The curvature of the cortex, as evident in the coronal FOV, was approximated by a slab geometry. (B) Following electrical stimulation of the left forepaw, we observe an increase in total hemoglobin concentration in the right hemisphere. An average axial slice through the maximum layer and its two neighboring axial slices is masked by a manually-determined brain mask and overlaid on a grayscale image of the mouse head. Coronal slices through the peak voxel in the axial slice show the evoked response up to 2mm beneath the surface of the scalp. (C) The average temporal response of voxels >50% of the peak response were used to calculate time courses for HbO₂, HbR, and HbT. Magnetic resonance image of the mouse brain is courtesy of the Duke Center for In Vivo Microscopy [49], accessed via INCF Scalable Brain Atlas [50].
Specifically, evoked hemodynamic responses reveal an increase in HbO$_2$ and HbT and a decrease in HbR (Figure 2.8C), and were repeatable across mice (see Repeatability). Group averaged peak concentration changes of 4 μM in HbO$_2$, -2.5 μM in HbR, and 1.5 μM in HbT, were observed at a depth of 1700um beneath the scalp surface (Figure 2.8B).

**Repeatability**

The data shown in Fig. 2.8 are averages of runs across five mice. Averaging data only across each individual mouse instead of across the entire group shows good repeatability. Maps of the peak frame show an ability to resolve the expected increase in HbO$_2$ and HbT, along with the decrease in HbT, at the individual mouse level (Fig. 2.9). Evoked responses also follow the expected temporal behavior at the individual mouse level (Fig. 2.10).

**Figure 2.9.** Maps of evoked responses in individual mice. Runs with visually identified evoked responses are block averaged (A-E) and then averaged across all mice (F). For the five mice in this study, 15, 25, 30, 35, and 15 minutes of data were included for each mouse out of 35 min total. The group average is calculated after a two-dimensional translation of each mouse’s data to spatially align the maximum value of each activation across mice. DOT reconstruction artifacts, seen here mostly as vertical stripes, show where reconstruction algorithms could be further optimized to increase data quality.
Figure 2.10. Evoked response time courses. (A-E) Time traces of HbO₂, HbR, and HbT for each mouse (Mouse 1-5) are calculated by averaging the time traces of all pixels greater than 50% of the maximum response in Figure 7 and for the average across all mice (F). The relative increases in HbO₂ and HbT and decrease in HbR follow expected time courses and show good repeatability across mice. Error bars show standard error across runs.

2.3.6 Resting State Functional Connectivity
Although the structured illumination DOT system cannot detect spontaneous activity non-invasively with high enough signal-to-noise ratio to cleanly observe resting state functional connectivity networks, we can still validate this technique’s ability to measure resting state networks as a function of depth by collecting data in a mouse following scalp reflection. For this preliminary validation experiment, imaging was performed several days following surgery and affixation of a clear optical window (see Sec. 4.2.2 for further details on cranial window imaging procedures). Collection of resting state data followed the same procedure as evoked response data collection, except without the electrical stimulation applied to the forepaw. The mouse was imaged with 530nm illumination, with 40 square wave patterns (10 spatial frequencies at two phases and two orientations each) combining to provide an overall frame rate of 2Hz. All data processing followed the same pipeline as shown in Fig. 2.3, until spectroscopy, as single
wavelength data does not permit solving the linear system (Eq. 1.5) to convert changes in absorption coefficient to changes in oxy- and deoxy-hemoglobin concentration. Hence, resting state functional connectivity maps were calculated as described in section 1.2, except using time traces of changes in absorption coefficient for determining correlation coefficients.

Figure 2.11. Resting state functional connectivity as a function of depth. (A) Typical functional connectivity maps in a mouse generated by planar optical intrinsic signal imaging data [15]. Seeds (black dots) are placed in the motor, somatosensory, and retrosplenial cortices. (B) Equivalent resting state functional connectivity data from the structured light diffuse optical tomography system. Connectivity maps are calculated throughout the volume by correlating the time traces of changes in absorption coefficient at every voxel with the changes at a seed location placed in the 400μm layer. Networks are seen to dissipate as a function of depth depending on their location within the cortex.

Comparing resting state networks between a mouse imaged using standard planar imaging techniques (Fig. 2.11A) and one imaged with structured illumination DOT (Fig. 2.11B) shows qualitatively similar results. In particular, the spatial extent of functional networks is consistent, although more focal in structured DOT data, with the expected bilateral correlations and anterior-posterior anti-correlations revealed using DOT. Further, DOT is capable of generating these networks as a function of depth, by looking at correlation maps in axial slices of deeper layers with seeds placed more superficially. This reveals a dissipation of networks as a function of depth, with the retrosplenic network fading first at a depth around 1.3mm, followed by motor
cortex at 1.6mm, and finally somatosensory cortex around 1.9mm. This could be reflecting the three-dimensional structure and curvature of the mouse brain, as the cortical surface extends deeper into the head further away from midline (see Fig. 2.8A).

2.4 Discussion
In this work, we developed a structured illumination approach to DOT for non-invasive imaging of brain function in mice. The structured illumination DOT (SI-DOT) system combined the dense spatial sampling of camera-based DOT systems, finite element light modeling (common with fiber-based DOT), and the rapid scanning afforded by structured illumination. To manage data quality, we developed several quality assessments to optimize the system and capitalize on its flexibility while also improving model accuracy by empirically calculating estimates of baseline optical properties. Feasibility was established by observing functional activations in vivo non-invasively, and by reconstructing their depth as well as their lateral position. While there are extensive reports of optical imaging of functional activations in mice with removal of either the scalp or both the scalp and skull, non-invasive optical mapping of functional responses in mice has not yet been widely reported or explored.

A primary strength of the SI-DOT system is its great flexibility with regard to the source and detector grid density. However, the potential complexity of SI-DOT data sets presents challenges in evaluating data quality. SNR is of central importance in DOT system design and data quality optimization prior to image reconstruction [26], [41]. More specifically, the relationship between light level and source-detector distance guides many decisions throughout the construction of the instrument, including optics, sensor locations and sizes, and exposure times or frame rates. As a result, the ability to assess measurement quality before image reconstruction is important to instrument development and optimization. Thus, we developed a method for evaluating SI-DOT
measurements using an effective distance between a detector pixel and an arbitrarily complex structured illumination pattern. We have found that this metric enables the optimization of SI patterns and aids in identification and removal of bad or noisy measurements. Together, these techniques allow for efficient optimization and balancing of imaging speed, resolution, and FOV. In this work, we optimized for a single view of the dorsal surface of the mouse head, consistent with our previous planar imaging systems [15], and imaged a FOV of 12x12x2mm using 2 wavelengths and 40 illumination patterns.

2.4.1 In Vivo Non-Invasive Activations

Our DOT imaging techniques allow us to reconstruct hemodynamic activity up to 2mm beneath the scalp surface. In response to electrical stimulation of the forepaw, we observe peak activity between 1.3 and 1.7mm beneath the surface of the scalp, a finding consistent with the expected depth of activity given structural mouse studies examining the thickness of the scalp, skull, and cortical layers [51]–[53]. The reconstructed images of HbO$_2$, HbR, and HbT have magnitude, temporal response, and axial location that are consistent with previous functional imaging studies [19], [54]. As a function of depth, the SI-DOT images show an increase in HbO$_2$ and decrease in HbR corresponding to somatosensory layers 2-4. This depth-dependent hemodynamic activity is reasonably consistent with observations from invasive thinned-skull preparations in rats using multispectral imaging [55] and Laminar Optical Tomography [56], and non-invasive functional MRI studies in mice [57], [58].

The penetration depth and resolution of the system can be approximated by reconstructing theoretical tissue structures using the system’s sensitivity matrix (see Sec. 3.3.3). First, we generate simulated measurements for a point activation. This data is then reconstructed using an inverted sensitivity matrix with regularization that reflects the operational SNR found in our
experimental data. Taking the FWHM to be the point-spread function, this yields an axial
resolution of 1.13mm and a lateral resolution of 1.11mm at a depth of 1.3mm. Similarly, we
quantified the depth sensitivity using a flat field imaging test by reconstructing a uniform
perturbation throughout the volume [47]. The sensitivity drops to 50% at a depth of 2.5mm,
setting a practical limit on the penetration depth of SI-DOT as constructed.

2.4.2 Context to Literature
Structured illumination addresses a critical issue that limited either the speed or spatial sampling
of previous DOT techniques. A similar strategy has been applied in microscopy, where the use of
structured illumination has improved the speed and resolution of fluorescence microscopy
beyond what was previously possible [48], [59], [60]. In related mesoscopic imaging work,
spatial frequency domain imaging (SFDI) utilizes structured illumination patterns of varying
spatial frequencies to probe different depths and provide 3D information [28], [29], [61], [62].
However, thus far the structured illumination literature has been limited to either applications
outside of brain imaging, or brain imaging that is invasive and/or without tomographic
reconstructions. Further, the relationship between source-detector distance and measurement
SNR, which is central to assessing image quality in traditional point illumination DOT, has not
been addressed using SFDI.

The primary application of wide-field structured illumination has been for clinical non-invasive
and depth-resolved assessment of skin and breast tissue in humans [63]. Although the technique
can provide 3D tomographic reconstructions of absorption heterogeneities [61], [62], thus far the
implementations in rodent neuroimaging, for example to monitor stroke [64], cortical spreading
depressions [65] or Alzheimer’s Disease [66], [67] have required invasive surgeries prior to
imaging or have not been used in applications with fast dynamics (~1s). Additionally, while
SFDI is commonly used to assess baseline optical properties [68]–[70], our technique compares the light fall-offs as a function of effective distance in the optical measurements versus a finite element forward model, which will be easily translatable to calculating optical properties in complex geometries.

2.4.3 Limitations and Potential Improvements
In this work, there are three assumptions in the forward model that potentially limit the accuracy of reconstructions: homogeneous optical properties, geometrical boundaries of the mouse head, and the use of the diffusion approximation. Even though the absorption and scattering properties of different types of biological tissues are in fact relatively homogeneous [71], the accuracy of the forward model could be increased by modeling the scalp, skull and brain separately. Additionally, although the scattering coefficient in tissue is relatively constant [72], implementing a wavelength-dependent power law [18] to uncouple absorption and scattering would further improve the accuracy of the forward model. Further, an iterative procedure using determined optical properties to calculate new fits of signal vs. effective source-detector separation, instead of assuming a μ_{eff} of 1 mm⁻¹, might allow for better empirical optimization of both optical properties.

The localization accuracy of the functional response depth could be increased by using a cylindrical head model, instead of the currently used slab geometry, or still further improved by using an anatomically-based head model derived from magnetic resonance imaging with co-registration and boundary identification, as is done in human fiber-based DOT imaging [26]. The SI-DOT system could achieve boundary identification in an automated and data-driven way by including a pattern sequence with point illuminations for surface profiling. Accurate anatomical head modeling could enable transformations of the DOT data to a common atlas space for group
comparisons of functional networks, as done previously for DOT studies in humans [26], [73], [74], while also accurately modeling both the boundary and the different layers of the mouse head.

Additionally, there is a slight model mismatch due to using a slab geometry to model the mouse head, which is curved. Because we constrained imaging to the top central portion of the skull, we estimate that there is approximately a <1mm displacement across the 10mm FOV. Given this curvature of the mouse head (Fig. 2.8A), our calculations of illuminated pixel locations and distances near the edge of the FOV may be inaccurate up to ~8% due to our use of a slab instead of a cylindrical geometry, according to simple trigonometry. Due to the exponential weighting of large distances in the effective source-detector separation calculation, the subsequent maximum error in effective distance is ~7%. While small, if this error were used in baseline measurements [75], it would introduce significant image errors. However, our application is the imaging of differential brain activity, and these model errors are divided out through the use of the Rytov approximation [76], [77]. Anatomical head modeling will allow for more precise calculations of both effective source-detector separation and point illumination locations in the forward model.

In this study we have used the diffusion approximation, whereas the radiative transport equation, as solved with Monte Carlo methods for example, would in principle be more accurate. Indeed a number of groups have shown similar reconstruction methods using Monte Carlo-generated Green’s Functions [78], [79]. While model errors are generally minimized by using ratiometric data (in this study the log-ratio data for temporal responses), the incorporation of Monte Carlo-derived forward models may provide better fits to the light intensity fall-off curves and better image quality.
Further exploration of parameter space might reveal ways to take further advantage of the system flexibility and optimize the trade-offs between imaging speed, resolution, and FOV. For example, shrinking the FOV would increase the camera speed and therefore increase the overall DOT frame rate or accommodate the addition of more source patterns while maintaining the same frame rate. Additionally, expanding the measurement set to consist of more spatially overlapping measurements and to include near infrared (NIR) wavelengths for deeper penetration have been shown to improve resolution and overall system performance in previous DOT systems [80]. This could be done in SI-DOT by utilizing multiple views, which would allow for a FOV covering the entire mouse brain (see Ch. 3). This combined with NIR wavelengths would take advantage of greater source-detector separations to probe deeper into the brain with a resolution of ~1/3*depth [81], or slightly better at shallower depths [56]. Improving resolution and sensitivity would also improve the system’s ability to perform non-invasive resting state imaging. Consequently, functional connectivity maps created from changes in oxy- and deoxy-hemoglobin could be directly compared to those derived from planar imaging techniques, allowing reiterations of previous studies of disease [22], [23], but using DOT to extend observations of these functional deficits to higher order and potentially sub-cortical brain networks.

2.5 Summary/Conclusion
Structured Illumination Diffuse Optical Tomography, with asymmetry between sources (few) and detectors (many), addresses a data rate limitation of previous DOT systems for rodents. SI-DOT improves upon current planar methods, which lack depth profiling and require surgical removal of the scalp, to provide non-invasive three-dimensional information of the brain. The SI-DOT system reported is built on an infrastructure with great flexibility. The techniques
developed for assessing the quality of SI-DOT datasets before image reconstruction enable efficient optimization of the system to balance imaging speed, resolution, FOV and computation time. Further, these techniques permit data quality analysis and an assessment of light model accuracy. The non-invasive three-dimensional imaging of the mouse cortex provided by SI-DOT has the potential to yield new insights into the functional architecture of the mouse brain and provide new avenues for studying healthy brain development, aging, disease, and therapies.
Chapter 3: Whole-Brain DOT in Mice Using Multiple Camera Views

3.1 Introduction

Functional magnetic resonance imaging (fMRI) has been established as the gold standard for functional neuroimaging in humans. Although recent studies have shown great improvements in the ability of fMRI to map functional brain networks in mice [82], [83], the technique is still mostly limited to dedicated neuroimaging centers due to its prohibitive cost and space requirements. Additionally, fMRI is primarily sensitive to changes in blood oxygenation via the blood-oxygen-level-dependent signal [84], and cannot easily be used to monitor biological contrasts in the brain besides hemodynamics, such as energy consumption or calcium dynamics.

Optical planar imaging techniques, such as optical intrinsic signal imaging (OIS, see Sec. 1.2), have demonstrated an ability to observe functional brain networks in mice using a wide range of contrasts [15], [85]–[88] (see Ch. 4). However, these traditional planar techniques are limited by minor invasiveness, typically requiring scalp reflection and possibly skull thinning, in addition to being restricted to a two-dimensional view of the cortex which provides no depth information. As a result, some brain networks or regions, such as the default mode network [89] and other clinically relevant higher order brain networks, are completely inaccessible.

Diffuse optical tomography (DOT) solves many of the problems limiting traditional planar techniques. Optical tomography utilizes functional near-infrared spectroscopy techniques, which can probe up to 2-3cm beneath the surface of the scalp and attribute light fluctuations to specific locations based on a numerical model of light transport in tissue [24]. This technique had previously been expanded to rats for neuroimaging [25] and mice for molecular fluorescence.
imaging [27], but only recently into mice for neuroimaging, by using structured pattern illumination to overcome the limitations of the previous techniques [90] (see Ch. 2).

Our previous structured illumination diffuse optical tomography method was somewhat simplified and limited. In particular, the numerical model of light transport required for tomographic reconstructions treated the mouse head as a slab geometry, which failed to account for the curvature of the head and possibly caused inaccuracies in depth localization.

Additionally, although this technique was capable of generating volumetric images, the use of a single projector for illumination and a single camera for detection had a limited range of source-detector separations and therefore a limited depth sensitivity. The off-the-shelf projector in use was restricted to illumination with built-in light emitting diodes (LEDs) with fixed wavelengths of red (626nm), green (523nm), and blue (455nm) that even under ideal conditions cannot easily probe deeper than ~5mm beneath the surface of the scalp (see Sec. A.2.2).

To address these limitations, we have expanded our structured illumination system to include three cameras for multiple views, and two custom-built light engines and projectors for multiple illumination angles. These custom projectors accommodate the use of arbitrary wavelengths and increased source-detector separations to provide better depth resolution and sensitivity. In particular, the custom projector and light engine support the use of both visible and near-infrared wavelengths for illumination, effectively providing a single system with the ability to image over multiple resolution scales. The system also incorporates in-situ surface profiling techniques to generate a unique surface boundary for each individual mouse, which provides far more accurate anatomical head modeling than previous slab-based methods. Because the system has no moving parts, surface profiling and in vivo functional brain imaging occur in quick succession. The remainder of this chapter will discuss the design and capabilities of this multi-view system, in
addition to preliminary simulation studies that demonstrate its imaging capabilities and provide an efficient framework for future optimization.

3.2 Methods

3.2.1 Multi-View Structured Illumination System

The multi-view structured illumination diffuse optical tomography system consists of three cameras and two projectors, distributed evenly around the upper hemisphere of the imaging plane (Fig. 3.1A). The cameras (Andor Zyla 5.5, Andor Technologies) are oriented at -60°, 0°, and +60° with respect to vertical, and are all focused to an overlapping imaging plane using 85mm f/1.4 camera lenses (Rokinon).

Figure 3.1. Multi-view structured illumination DOT system. (A) System schematic showing the relative positions of the three cameras and two projector sources, with sCMOS and digital micromirror device (DMD) chips radially aligned. (B) Custom light engine design, showing six different wavelengths of LEDs and singlet lens selections for each to minimize power loss before entering the liquid light guide that carries light to the projector. (C) The six wavelengths of the light engine shown over the attenuation spectra of oxygenated and de-oxygenated hemoglobin. The visible wavelengths provide good resolution at shallow depths, while the near infrared wavelengths provide better depth sensitivity. (D) Custom optics adapted from the Texas Instruments Lightcrafter 4500 projector take the output from the light engine and project onto a DMD chip to generate structured illumination patterns. (E) Fields of view of the three cameras and illumination areas of the two projectors, shown as an example on the face of a 3D printed action figure. (F) A sample structured light pattern from each projector shown from the perspective of each camera.
A custom light engine was assembled combining 6 light-emitting diodes (LEDs, Mightex Systems) and their appropriate dichroic mirrors to merge them into a single path. Additionally, each LED has an individually selected singlet lens depending on the distance its light must travel through the light engine (Fig. 3.1B). Focal lengths of singlet lenses were chosen by simulating ray tracing using optical design software (Zemax) and selecting lenses that minimize power loss through the light engine (see section A.3.1). For optimal spatial arrangement of system components, each projector has its own light engine, with a liquid light guide (Mightex Systems) transporting the light from the vibration-isolated light engine to the projector mounted within the system. The six wavelengths include a visible arm, with wavelengths of 470nm, 530nm, and 625nm, and a near-infrared arm with wavelengths of 680nm, 740nm, and 850nm. The visible wavelengths are more highly attenuated [14] and therefore provide good contrast and resolution at shallower depths, while the near infrared wavelengths are less attenuated and can therefore penetrate deeper and provide greater depth sensitivity (Fig. 3.1C). This combination of wavelengths is crucial to providing imaging sensitivity throughout the full volume of the mouse head.

In order to accommodate the output of the liquid light guide from the custom light engine, we assembled a custom projector modeled after the Texas Instruments Lightcrafter 4500 used in the preliminary system. Critical components of the Lightcrafter 4500, such as the digital micromirror device (DMD) chip (for generating the structured light patterns), the total internal reflection (TIR) prism (for redirecting light patterns from the DMD out of the projector), and any necessary electronics, were removed and assembled in a 3D-printed frame that allowed custom optics to bring the liquid light guide to the necessary position for proper focusing and DMD illumination (Fig. 3.1D, see section A.3.2). Briefly, light exiting the liquid light guide is collimated and then
focused down through the TIR prism for uniform DMD illumination. Structured light patterns reflect off the DMD and back through the TIR prism, where light is reflected out of the projector. This custom projector is mounted to a vertical breadboard angled towards the imaging plane, where a camera lens focuses the structured light pattern onto the object being imaged.

A 3D-printed action figure with facial size and curvature very similar to that of a typical mouse head was used throughout system construction and optimization for optical alignment and system timing tests. The projectors are oriented such that ~20% of their respective illumination areas on the 3D printed action figure overlap (Fig. 3.1E). The cameras are oriented such that ~50% of the detectors from each of camera 1 and camera 3’s fields of view overlap with camera 2’s field of view.

3.2.2 Multi-View System Processing Pipeline
The full process of generating and collecting measurements with the multi-view tomography system, and ultimately converting them to brain hemodynamics (Fig. 3.2), is an expansion of the processing pipeline from the preliminary single-view system (see Sec. 2.2). Illumination patterns of interest are designed in Matlab and loaded into the onboard memory of the projector using the Texas Instruments software for the Lightcrafter 4500, after which our projector is based. Voltage triggers to control camera frame collection, structured pattern display, and LED selection are custom designed in Matlab and sent to the system using a Texas Instruments Data Acquisition card. A typical scan sequence progresses through all spatial frequencies for a given wavelength first, then scans through all wavelengths for a given projector. This sequence is then repeated on the second projector.

Before carrying out structured illumination imaging, a 20x20 grid of point illumination “patterns” is sequentially scanned using 530nm light to perform surface profiling. First, a series
of calibration scans is performed if it has not yet been done for the active system set-up. The grid of points is scanned over a flat surface parallel to the imaging plane for each projector-camera pair. This is repeated at multiple heights for each pair to calibrate the surface locations at each height with their corresponding camera sensor locations (Fig. 3.3A). This set of calibration scans is sufficient for all subsequent surface profiles, until system alignment changes, at which point a new set of calibration data is required.

**Figure 3.2.** Multi-view tomography system processing algorithms, with crucial elements bolded. Raw data are collected as either point-scanned measurements for surface profiling (right, orange boxes) or structured illumination measurements for brain imaging (left, red boxes). Point illumination data is used for in situ calculation of the mouse head boundary and assignment of source and detector locations on the head surface for light modeling (center, brown boxes), which uses NIRFAST to generate a model of light diffusion in tissue and a sensitivity matrix for each wavelength. This sensitivity matrix is inverted and multiplied by an optimized list of measurements to reconstruct brain hemodynamics (bottom, blue boxes).

With calibration of the current system set-up complete, surface profiling of the sample of interest commences by scanning the same 20x20 grid of points over the surface so that each point’s position can be assigned a location in each camera’s coordinate frame (Fig. 3.3B). This process produces a point cloud (Fig. 3.3C), which can subsequently be converted into a surface mesh (Fig. 3.3D), for each individual projector-camera pair. In order to generate a surface mesh of the full volume, as is necessary for accurate anatomical forward modeling of light transport, the
individual surface profiles must be co-registered. Using the independent coordinate frames for each camera and the subset of points from each projector that is visible by multiple cameras (Fig. 3.4A-C), an affine transform is calculated to convert each camera’s coordinate system into a global frame of reference, which is defined to be the coordinate system of camera 2.

**Figure 3.3.** Surface profiling and 3D surface capture for projector camera pairs. (A) For calibration, point illumination patterns are projected onto an adjustable flat surface parallel to the camera lens focal plane at multiple known heights. Source beams’ directions and positions are computed from camera lens properties (e.g., focal length) and the different positions of the illumination point in each image. (B) The same point illuminations are projected onto the surface of an object of interest at the beginning of an imaging session. For each point pattern, the intersection of the reemitted beam (computed from lens properties and image) and source beam provides the location of a point on the head surface. (C) A 3D point cloud showing the location of each point on the surface of a 3D printed action figure’s face in a single camera’s coordinate system. (D) The 3D point cloud is converted into a mesh model of the mouse head surface, with individual surface locations sampled by each individual camera pixel shown.
Figure 3.4. Co-registration of multiple camera views. (A) Some illuminated locations on the surface, like the magenta point shown, reemit light into multiple cameras and are therefore “seen” by multiple cameras. (B) Points seen by both Cameras 1 and 2 are shown in magenta. These overlapping points are used to solve for the parameters of an affine transform that allows position coordinates in camera 1’s frame to be calculated with respect to camera 2’s frame. (C) Points seen by both cameras 2 and 3 are similarly used to solve for the affine transformation from camera 3’s frame into camera 2’s frame. (D) Affine transformations are applied between each pair of camera frames in sequence as needed to express all point locations into a single camera’s frames, which is used as the global 3D coordinate system.

After surface profiling, structured illumination measurements are collected. Scans typically last for 5 minutes, with anywhere from 6 to 20 spatial frequencies and 3 to 6 wavelengths used per projector. Structured light measurements can be assigned locations on the head surface using the coordinate frames established during surface profiling. Prior to reconstruction, a series of pre-processing steps are carried out to identify and remove noisy or otherwise inaccurate measurements, consistent with our previous methods (see Ch. 2). The logmean (equation 2.4) of the set of good measurements is then calculated to convert raw light level measurements into differential changes. Measurements with a short effective source-detector separation are
considered superficial (non-brain) measurements, and have their signal regressed from the full measurement set prior to reconstruction.

Forward modeling follows the same procedure as our previous work (see Sec. 2.2.2), but now uses the in-situ surface profile to assign proper locations and angles of incidence for each source and detector on the head surface. Any points visible by more than one camera, as identified when calculating the affine transform for surface profile co-registration, are counted just once to avoid having excessive fluence assigned to these locations during forward modeling. Structured illumination pattern boundaries are empirically calculated as done previously, with point Green’s functions corresponding to the points determined to be illuminated (with the exception of those excluded due to being visible by multiple cameras) summed together to form that source pattern’s Green’s function. Green’s functions are calculated for each source (structured pattern) and detector (camera pixel) as before, with the sensitivity matrix and subsequent reconstruction carried out independently for each wavelength. Reconstructed data, representing differential changes in absorption coefficient, are filtered to the frequency band of interest, spatially smoothed, and a mean signal over the whole brain volume is regressed to remove any contamination by global hemodynamic fluctuations. Finally, spectroscopic unmixing using the different wavelengths converts the reconstructed data into differential changes in oxygenated and deoxygenated hemoglobin.

3.3 Results

3.3.1 Surface Profiling
In order to quantify the accuracy of our surface profiling technique, we scanned the 3D-printed action figure, both due to its convenient size and because we can compare our system-derived surface profiles with the expected surface given a mesh generated from the original model. Once
a complete surface profile and corresponding surface mesh are generated (Fig. 3.5A), we can quantify errors between overlapping points for each pair of cameras (camera 1-camera 2 and camera 2-camera 3), between all points on the surface across two consecutive runs, and between all points on the surface for our mesh and that generated from the original 3D model.

Figure 3.5. 3D mesh error estimation. (A) A full 3D surface mesh is generated from the affine transformed points from each camera-DMD pair. (B) The disagreement in spatial location between each pair of overlapping points is displayed as the Euclidian distance between the points, as determined by each individual camera. (C) Two runs of quick succession on the same surface generate a pair of surface profiles. Spatial error between each individual point over the surface between the two runs provides a representation of the contribution of photon noise in the surface calculation. (D) By transforming the surface to overlay on the original 3D model used to generate the print of the action figure, the overall error with respect to a “gold standard” can be determined. (E) Average error across all points is shown for each of the four cases.

Of the 400 points in the 20x20 grid from a single projector, 262 are shared between cameras 1 and 2. These points have an average error, calculated as the average disagreement in spatial
location by Euclidian distance, of 0.17 ± 0.10 mm. Cameras 2 and 3 share 201 points, with an average error of 0.18 ± 0.12 mm (Fig. 3.5B).

Consecutive identical runs reveal an average error among all points between all four projector-camera pairs of ~0.02mm, with about half of the points having a test-retest error of less than 0.01mm (Fig. 3.5C). This demonstrates the relatively insignificant errors produced by Poisson noise on a run-to-run basis, suggesting that our signal-to-noise ratio is not a limiting factor in generating accurate surface profiles. Finally, to compare the quality of the surface mesh to that which is generated from the original 3D model, we transform the two point clouds to be spatially aligned. Because the 3D model has a much denser point cloud than that generated by our surface profile, we only consider errors between each surface profile point and its corresponding nearest neighbor in the original 3D model. For all of these points, the average error is ~0.4 ± 0.2mm, with a majority of the points with errors greater than 0.5mm falling at the extreme edges of the field of view (Fig. 3.5D).

3.3.2 Illumination Pattern and Measurement Optimization
In order to assess measurement quality, we must first assign each individual source and detector to its proper location on the mouse mesh surface. Pattern boundaries are determined empirically by labeling all pixels greater than 15% of the maximum in a given frame as “on” for a given illumination pattern (Fig. 3.6A, middle row). To avoid double counting values in the exponentially weighted average, any surface head locations shared by either camera 1 or camera 3 with camera 2 are removed (Fig. 3.6A, bottom row). This is also necessary to avoid giving improper weights in the forward model to sources which are visible to more than one camera.
Figure 3.6. Measurement 3D location assignment and optimization. (A) A white light image of a sample pattern is shown from each camera’s perspective (first row). Each frame is masked at 15% of the maximum value to empirically determine boundaries of the illumination patterns (second row). Illumination locations with multiple detectors collecting light are masked so that incident light is not double counted when calculating the forward model (third row). (B) The sample pattern is shown with its illumination location on the surface of the mouse head mesh highlighted in yellow. (C) The effective source-detector (SD) separation for every non-illuminated detector location on the surface of the mouse head mesh is calculated for the sample pattern. (D) Measurement density as a function of effective source-detector separation for the full measurement set, and the equivalent measurement set using only components of the previous SI-DOT system. (E) Signal fall off as a function of effective source-detector separation for each of the six wavelengths, averaged at each unique distance. Measurements with a signal below the average per-pixel standard deviation of the background frames (orange box) are removed.
Source illumination patterns are defined as the combination of points included in this binary mask across all three cameras, and can be assigned to their respective locations on the mesh surface using the coordinate systems established during surface profiling (Fig. 3.6B). The effective source-detector separation is calculated between each illumination pattern and each dark detector location on the surface of the head for that pattern using the same method as previously discussed (see Sec. 2.3.1); briefly, the effective distance is an exponentially weighted average of the distances between a given dark pixel to every illuminated pixel in that pattern (Fig. 3.6C).

By grouping measurements into 0.1mm effective distance bins, we can see the density of measurements as a function of effective source-detector separation (Fig. 3.6D). For the full multi-view measurement set, the shallowest depths have \( \sim 10^4 \) measurements, while most distances up to an effective distance of 10mm have \( 10^3 \) or slightly fewer measurements each (Fig. 6D, blue). For the equivalent set of measurements limited to just the single camera and projector used in the previous single-view DOT system (Ch. 2), there are only \( \sim 10^2 \) measurements at each distance up to 5mm, with very low measurement density beyond 5mm, including no measurements beyond an effective distance of \( \sim 11 \)mm (Fig. 6D, orange). The average of all measurements within a given 0.1mm bin shows that the signal as a function of effective distance spans at least 4 orders of magnitude before getting into the noise floor of the system, with the exact dynamic range varying as a function of wavelength (Fig. 6E). The noise floor provides an empirical threshold for removing noisy measurements prior to reconstruction, and is calculated to be the average standard deviation across the background (dark) frame.
3.3.3 Light Modeling Accuracy, Sensitivity, and Resolution

Light Model Optimization

To assess model accuracy, we can use the Green’s functions generated from the forward model to plot predictions of signal fall-off as a function of effective source-detector separation and calculate the agreement between this fall-off and the true fall-off for that wavelength. Comparing the predicted signal (Gsd, see Sec. 2.2.2, Fig. 3.7A) and the normalized raw signal (Fig. 3.7B) over the whole surface of the mouse head for an example spatial frequency at 680nm illumination qualitatively shows good agreement over the majority of the field of view, with the exception of the edges of the FOV where inhomogeneities such as unshaven fur may affect the raw data. As in our previous work (see Sec. 2.3.3), we can modify the optical properties governing the forward model until error between the signal vs. effective source-detector separation fall-off curves is minimized, as demonstrated for 680nm illumination (Fig. 3.7C).

![Figure 3.7](image)

**Figure 3.7.** Forward model quality assessment. (A) The forward model’s prediction (Gsd, the denominator of the sensitivity matrix) for light levels across the surface of the mouse head from a given illumination pattern at 680nm. (B) Normalized in vivo raw data as detected by the multi-view imaging system for the same illumination pattern and wavelength. (C) Signal fall-off as a function of effective source-detector separation for both the predicted and raw data, including all structured patterns, for 680nm illumination.

We found the absorption coefficients that maximize the agreement for 470nm, 530nm, 625nm, 680nm, 740nm, and 850nm illuminations respectively to be 2.03cm$^{-1}$, 2.39cm$^{-1}$, 1.63 cm$^{-1}$, 1.15cm$^{-1}$, 0.83cm$^{-1}$, and 0.16cm$^{-1}$ (Fig. 3.8). Direct comparisons of the fall-off curves of
predicted data and raw data permit the use of the same model mask as discussed in section 2.3.4, where measurements whose counts deviate from the expected value by a significant amount can be removed prior to reconstruction.

![Figure 3.8. Optimized optical properties for all six wavelengths. Signal fall-off as a function of effective source-detector (SD) separation for predicted data and raw data for both visible wavelengths (2.03 cm$^{-1}$ for $\lambda = 470$ nm (A), 2.39 cm$^{-1}$ for $\lambda = 530$ nm (B), 1.63 cm$^{-1}$ for $\lambda = 625$ nm (C)) and near-infrared wavelengths (1.15 cm$^{-1}$ for $\lambda = 680$ nm (D), 0.83 cm$^{-1}$ for $\lambda = 740$ nm (E), and 0.16 cm$^{-1}$ for $\lambda = 850$ nm (F)). Significant deviations between the raw data and the model can be seen at large source-detector separations, providing a mask for measurement removal prior to reconstructions.](image)

**System Sensitivity and Resolution**

Ideally, system resolution and sensitivity could be measured and subsequently optimized by collecting in vivo data of a known or expected outcome, such as electric stimulation of the forepaw, as was done in our previous work (see Sec. 2.3.5). However, given the computationally intensive demands of the large measurement sets provided by the multi-view system, reconstructions of simulated data can be used to assess system quality much more efficiently.

Having generated sensitivity matrices with the optimized optical properties for each wavelength, simulated measurements ($y$ in $y = Ax_{sim}$, see Eq. 2.3) can be calculated by multiplying the
optimized sensitivity matrix ($A$) by a vector of simulated data ($x_{sim}$). Then, reconstructed simulated data can be calculated by inverting $A$ (with Tikhonov inversion parameters of $\lambda=0.01$ and $\beta=1$, following the notation of Dehghani et al. [47], see Sec. 2.2.2) as in equations 2.6 and 2.7, and multiplying this inverted sensitivity matrix by the previously calculated simulated measurements:

$$x_{calc} = A^#(Ax_{sim})$$

Comparisons between $x_{calc}$ and $x_{sim}$ can provide information on system performance, in addition to providing a means for optimizing the reconstruction parameters that generate $A^#$. For example, the point spread function can be estimated at different locations throughout the volume by creating an $x_{sim}$ to be zeros everywhere except for a single point perturbation at the location of interest. In this case, the reconstructed $x_{calc}$ represents the point spread function, or resolution, at that location for the sensitivity matrix (or, equivalently, the illumination wavelength) used in the simulated reconstruction.

To assess performance in the multi-view system, we reconstructed point perturbations in the center of the volume in the x-y plane at a range of different depths (Fig. 3.9A). Resolution and depth localization accuracy can be qualitatively observed as a function of depth for each wavelength by looking at axial slices through the volume in the layer in which the original perturbation was placed (Fig. 3.9B). The shorter wavelengths in the visible part of the spectrum (470nm, 530nm, 625nm) appear to have slightly better resolution at shallow depths over the near-infrared wavelengths (680nm, 740nm, 850nm), while the near-infrared wavelengths have better resolution at greater depths (Fig. 3.9C). Further, the visible wavelengths are not capable of successfully reconstructing the point perturbations at depths greater than ~5mm.
Figure 3.9. Point perturbation simulation reconstructions. (A) Point perturbations are placed in the center of the volume in the x-y plane at 6 equidistant depths ranging from ~0.5mm to ~8mm beneath the surface. (B) Axial slices through the volume at each perturbation depth are used for point-spread function visualization. (C) Reconstructions of point perturbations from each of the six wavelengths’ sensitivity matrices, at each of the six layers of interest. Images are scaled from 0 to the maximum value in that whole volume’s reconstruction. All wavelengths have good resolution at shallow depths, with visible slightly outperforming near-infrared. At greater depths, visible wavelengths are not able to localize the original perturbation, while longer wavelengths still show a blurred reconstructed point.
A similar analysis can be used to assess system sensitivity in addition to system resolution. Instead of reconstructing a point perturbation to determine the point spread function at that location, the sensitivity can be estimated via a single reconstruction of a uniform perturbation throughout the volume. In this case, we set $x_{sim} = 1$ in Eq. 3.1 at every voxel location, and observe the uniformity of the reconstructions at each layer (Fig. 3.10). Looking at axial slices of the same subset of layers as previously, we can see the relatively uniform sensitivity as a function of depth for all 6 wavelengths up to a depth of ~5mm (Fig. 3.10, top 4 rows).

**Figure 3.10.** Multi-view tomography system sensitivity. Reconstructions of a uniform perturbation of 1 throughout the volume for each wavelength show the system sensitivity as a function of depth. Axial slices are at the same depths as illustrated in Fig. 3.9B. Values close to 1 represent good sensitivity, while values significantly less than 1 represent a lack of sensitivity of that wavelength in that location.
At the greatest depth of ~8mm, only 850nm illumination is still sensitive (defined to be where sensitivity is >~50%) to changes at the innermost regions of the volume (Fig. 3.10, bottom row). At these larger depths, some sensitivity is preserved near the boundaries from all illumination wavelengths, as these locations are still within a few millimeters of the exterior surface. Note that the inconsistent number of voxels visible in the most superficial layers as compared to deeper layers is due to the mouse head not being perfectly parallel with the imaging plane (as illustrated by the slices in Fig. 3.9B).

Finally, to better quantify sensitivity and resolution throughout the volume, we can perform these reconstructions at each voxel layer, instead of just the select six shown in Figures 3.9 and 3.10. Resolution and sensitivity were calculated in 34 layers, each with a thickness of ~292μm, up to a depth of ~10mm beneath the surface. The sensitivity at each depth is calculated as the average of all pixels within the volume in that layer. All six wavelengths have a relatively uniform sensitivity up to a depth of ~5mm (Fig. 3.11A). The sensitivities of the three visible wavelengths begin to decrease rapidly after a depth of 5mm, falling below 50% sensitivity at a depth of ~7mm for all three. The near-infrared wavelengths, on the other hand, stay above 50% sensitivity until at least 8mm of depth, with the 850nm illumination remaining greater than 50% all the way throughout the volume.

The resolution of the multi-view tomography system is calculated as the full-width at half maximum of a line profile through the original point perturbation location (as shown in Fig. 3.9A for 6 of the 34 layers) in either the axial or lateral plane. We observe a lateral resolution roughly equal to 2/3*depth for the shortest wavelengths, with slightly worse resolution for the longer wavelengths, up to a depth of ~5mm beneath the surface (Fig. 3.11B). Flattening of resolution curves deeper than ~5mm is likely due to a lack of sensitivity in this region, and therefore is not
representative of the true resolution at these depths for all wavelengths. In the axial dimension, we observe a resolution roughly equal to the depth of the perturbation at all wavelengths (Fig. 3.11C).

**Figure 3.11.** Sensitivity and resolution of the multi-view structured illumination tomography system. (A) System sensitivity is calculated at 34 layers, each 292 µm thick, as the average value in each layer following the reconstruction of a uniform perturbation. (B) Lateral resolution is calculated as the full-width at half maximum of a line profile through the original location of the reconstructed point perturbation in the x-y plane. (C) Axial resolution is calculated as the full-width at half maximum of a line profile through the original location of the reconstructed point perturbation in the x-z plane.

### 3.4 Discussion

In this work, we have expanded our previous single projector, single camera diffuse optical tomography imaging system to incorporate three cameras and two projectors. The use of multiple views provides a combined field-of-view covering the entirety of the upper hemisphere of the mouse head, with large effective distances between sources and detectors permitting sensitivity to much deeper regions than the single camera/single projector system. Additionally, the custom built light engine offers illumination from six different wavelengths, three visible and three near infrared, which combine to provide good resolution at shallow depths while maintaining sensitivity at greater (sub-cortical) depths. The multi-view system also incorporates in-situ surface profiling, which generates a unique anatomical boundary for each mouse and much more accurate forward models of light transport in tissue for tomographic reconstruction than our
previous use of a slab geometry. Reconstructions of simulated data provide assessments of system sensitivity and resolution throughout the volume of the mouse brain; while the single view system’s sensitivity dropped below 50% at a depth of 2.5mm, which limited it to only cortical imaging, the multi-view system maintains good sensitivity up to several millimeters beneath the surface, demonstrating a capability of imaging higher order and sub-cortical brain networks and regions.

### 3.4.1 System Design

The multi-view structured illumination diffuse optical tomography system was designed to accommodate flexibility. While the off-the-shelf projector successfully imaged hemodynamic activity in mice (see Sec. 2.3.5), the custom-built projector and light engine allow the use of any LEDs or wavelengths. For example, higher powered LEDs around 470nm could be used for fluorescence calcium imaging (see. Sec. 4.3.1), and given the system design, LEDs can easily be exchanged with no further modifications necessary beyond possibly changing out a dichroic mirror. This efficient flexibility cannot be easily afforded in typical optical imaging systems.

Additionally, the use of sCMOS cameras as the detectors provides a flexible framework to optimize the tradeoffs between field-of-view and imaging speed, as they have a dense grid of detector pixels that can be binned or cropped to accommodate different imaging preferences.

The abundance of possible illumination patterns and wavelengths available does present a challenge however, as the relatively low (~100Hz) frame rates of the sCMOS cameras limits the overall speed of the system. At full fields of view with the overlapping measurements shown in Fig. 3.1E, if all six wavelengths are used, imaging speed is limited to ~0.33Hz. While these overlapping measurements are helpful for reconstructing tomographic data, they can be partially sacrificed by cropping the sensor along one dimension to increase frame rates. Though this may
decrease system sensitivity at large depths slightly, it does not affect the overall field of view, as only overlapping measurements will be removed. Therefore, the system accommodates sufficiently fast imaging to avoid aliasing inherent fluctuations such as heart or respiration rate.

Another potential limitation of system hardware is the significant distance between the LED dies at their emission and the imaging plane. The most distant LEDs in the light engine, 625nm and 850nm (see Fig. 3.1B), travel through three dichroic mirrors, the liquid light guide, and the custom optics in the projector before finally illuminating the digital micromirror device and exiting the projector as structured patterns directed towards the mouse head in the imaging plane. As a result, through several optical elements there is a lot of potential for light loss and subsequent decreases in power, possibly limiting applications to fluorescence imaging, which requires high-powered illumination for sufficient signal-to-noise ratio (SNR). However, recently developed high-powered LEDs, combined with careful construction of the custom optics and light engine using ray tracing software such as Zemax, should allow for sufficiently high-powered illumination to overcome any potential power loss (see Sec. A.3.1 and A.3.2).

3.4.2 Surface Profiling

The current scan parameters of the system allow for complete in-situ surface profiling to occur in just two minutes prior to standard structured illumination imaging. Given that a typical imaging session lasts 45-60 minutes, this is a relatively negligible amount of time in exchange for a unique forward model per mouse, with surface profiles accurate to within a few hundred microns for the majority of the field of view (see Sec. 3.3.1, Fig. 3.5). To further explore the performance of our surface profiling technique, multiple scans of the 3D printed action figure were carried out with a number of parameters varied to determine whether accuracy could be improved to closer to the voxel size (≈300μm) over the entire field of view. To improve the SNR at each individual
point of the scan, we investigated both slowing down the scan to increase camera exposure time per frame, and opening the camera lens aperture while keeping the scan time consistent.

Table 3.1 Surface profiling errors for various scan parameters

<table>
<thead>
<tr>
<th>Scan Type</th>
<th>Cam 1 vs Cam 2</th>
<th>Cam 2 vs Cam 3</th>
<th>Run 1 vs Run 2</th>
<th>Profile vs 3D model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.179</td>
<td>0.187</td>
<td>0.0111</td>
<td>0.411</td>
</tr>
<tr>
<td>Slow</td>
<td>0.177</td>
<td>0.183</td>
<td>0.0154</td>
<td>0.398</td>
</tr>
<tr>
<td>Open Aperture</td>
<td>0.210</td>
<td>0.208</td>
<td>0.0179</td>
<td>0.419</td>
</tr>
<tr>
<td>Blue (470nm)</td>
<td>0.203</td>
<td>0.205</td>
<td>0.0684</td>
<td>0.458</td>
</tr>
<tr>
<td>NIR (850nm)</td>
<td>0.208</td>
<td>0.227</td>
<td>0.0956</td>
<td>0.492</td>
</tr>
</tbody>
</table>

The slower scan, occurring over 4 minutes with twice the exposure time per frame, produced very slight (<5%) improvement, which was deemed insufficient to justify sacrificing more scan time. Opening the camera lens aperture such that the signal increased to approximately the same value as the longer exposure time experiment caused a decrease in surface profile accuracy. This is likely due to the algorithm requiring beams to be following a straight path between the center of each lens and the sample for accurate calculation of their intersection points (Fig. 3.3). A more open aperture allows light rays to travel along paths that do not pass through the very center of the lens, so any improvement in SNR is countered by less precision in the angle of incident beams. Surface profiles calculated by scans of 470nm and 850nm point illumination show significance decreases in accuracy as well. This could be caused by more intrinsic noise in the 470nm or lower power and therefore lower SNR, as is the case for the 850nm LED.

If mice could be imaged while awake (see Ch. 4), so that scan time is not limited by anesthesia duration, surface profiles could be improved slightly by taking longer scans with greater camera
exposure times, but overall our algorithm performs reasonably well compared to other similar surface profiling techniques [91], [92]. Optimization of surface profiling parameters would be further improved by calculating errors between meshes directly, as opposed to the nearest neighbor treatment currently used for assessing errors between our surfaces and the gold standard (Sec. 3.3.1). Additionally, the value of the surface profiling algorithms could be further increased upon development of a means of affine transforming the surfaces between mice to a common atlas-space, as is typically done in human tomographic imaging of the brain [26]. In this case, to avoid the computationally intensive task of generating and inverting the sensitivity matrix for each individual mouse, a single optimized average sensitivity matrix could be affine transformed to match the surface boundary of each mouse and used in subsequent reconstructions.

3.4.3 System Performance and Quality Assessments
We have developed a number of techniques both to assess measurement quality before reconstruction, and to assess overall system quality following reconstructions of simulated data. These tools combine to provide several avenues to ultimately optimize system quality for tomographic reconstructions of the functional architecture of the entire volume of the mouse brain. In particular, this system could be used for a more thorough empirical determination of baseline optical properties for forward modeling. This could possibly permit treating the volume as multi-layered instead of assuming homogeneous optical properties throughout, which could be afforded with such high measurement density over a large range of depths. Additionally, different sets of illumination patterns could be explored to determine the tradeoff between measurement density (Fig. 3.6D) and depth sensitivity/resolution (Fig. 3.11). If faster imaging speed is necessary, this will be a necessary task to determine how many or which spatial
frequencies can be sacrificed to increase imaging speed with as little effect on sensitivity and resolution as possible. These optimizations could be further improved by calculating sensitivity and resolution as a function of radial depth instead of along the z-axis, as this would prevent increased sensitivity near the boundaries currently create artificially inflated values at the greater z-depths (Fig. 3.11A).

The choices of inversion parameters, which so far has been based off previous human tomography literature [26], can also be explored to investigate their effects on reconstruction accuracy and resolution. With a highly optimized system, in vivo validation could be carried out by looking at evoked responses as before (see Sec. 2.3.5). Instead of just focusing on forepaw activations, the whole brain resolution and sensitivity could be tested and optimized by evoking responses in forepaw, hindpaw, and whisker barrel cortices, and determining how accurately each of these can be spatially resolved.

3.5 Conclusion
The multi-view structured illumination diffuse optical tomography system expands on our previous single camera, single projector system to provide a much greater field of view and overall flexibility. With complete flexibility over a wide range of imaging and processing parameters, in addition to a number of tools that have been built to efficiently assess the effects of these parameters on image quality, non-invasive whole-brain volumetric imaging of functional connectivity is within reach. This technique can be applied to a large variety of previously inaccessible areas of study, such as volumetric imaging of contrasts besides hemoglobin (Ch. 4), monitoring the infant mouse brain, and measuring deviations from healthy functional brain development in the presence of a developmental disease (Ch. 5).
Chapter 4: Awake Mouse Imaging

4.1 Introduction
While previous observations of resting state functional connectivity (FC) in mice have proven useful for a wide range of applications and disease studies [15], [22], [23], [36], [93], [94], a common planar imaging technique for such studies, optical intrinsic signal (OIS) imaging, has been limited to observing hemoglobin-based contrasts and is most commonly carried out in anesthetized mice. Studying hemodynamics in awake, behaving mice can reveal additional information about the functional architecture of the brain without the typical confound of anesthesia in standard resting state FC studies. Additionally, oxy- and deoxy-hemoglobin fluctuate significantly slower than and are several steps downstream from neural activity, the most direct and immediate brain response following a stimulus. A system with the flexibility to image multiple contrasts and types of connectivity besides just FC of hemoglobin dynamics would allow probing of multiple stages of the hemodynamic response, providing information more closely related to neural activity [95]–[99]. Further, imaging mice while awake and behaving as they normally would removes many confounds related to, while also allowing a number of studies comparing the awake, anesthetized, and naturally sleeping brain. This provides a more robust and clinically translatable assay for longitudinal studies of brain diseases and treatments in mice. We have developed a system to accommodate awake mouse imaging which is capable of probing brain function using different strategies beyond simple observation of hemodynamics, including fluorescence imaging of calcium dynamics in the brain and targeted optogenetic excitation of specific brain regions.
4.2 Methods

4.2.1 Awake System Hardware Development
The awake mouse imaging system is a revised version of our previously published optical intrinsic signal (OIS) imaging system [15], which has been modified to accommodate awake mouse imaging and the flexibility required for fluorescence calcium imaging and targeted optogenetic photostimulation. For image detection, we use a cooled, frame transfer EM-CCD camera (iXon, Andor Technology Ltd.) in combination with an 85mm f/1.4 camera lens (Rokinon, New York, NY). To increase frame rate as well as increase SNR, the CCD is binned at 4x4 pixels; this reduces the resolution of the output images from 512 x 512 pixels at full frame to 128 x 128 pixels, but allows for an increase in frame rate.

Light emitting diode (LED) wavelengths must be selected to provide calcium fluorescence excitation, distinguish between oxy- and deoxyhemoglobin absorption for oximetry, and enable correction of calcium fluorescence (GCaMP6 or G6, see Sec. 4.3.1) emission to account for confounding hemoglobin absorption. For this system, sequential illumination is provided by four LEDs (\(\lambda = 470\text{nm}, 530\text{nm}, 590\text{nm}, \text{and} 625\text{nm}, \text{Mightex Systems}\)), which combine to provide hemodynamic information via our typical spectral unmixing and additional contrast information depending on the contrast information (see Sec. 1.2). For fluorescence calcium imaging, the 470nm LED (peak measured \(\lambda = 454\text{nm}\)) is used for calcium excitation (see Sec. 4.3.1), while the 530nm, 590nm, and 625nm LEDs (peak measured \(\lambda = 512\text{nm}, 595\text{nm}, \text{and} 640\text{nm}\) respectively) are used for OIS imaging (Fig. 4.1). Additionally, the 530nm LED is used as an emission reference for fluorescence in order to remove any confound of hemodynamics in the fluorescence signal. A 515nm longpass filter (Semrock, Rochester, NY) is placed in front of the CCD to filter out 470nm fluorescence excitation light, while a 460/60nm bandpass filter
(Semrock) is used in front of the excitation source to further minimize leakage of fluorescence excitation light through the 515nm longpass filter.

**Figure 4.1.** Concurrent GCaMP fluorescence and OIS imaging system. (A) GCaMP6 dynamics are imaged using fluorescence measurements, wherein the fluorophore has a peak excitation at 497nm and peak emission at 512nm. A longpass filter at 515nm is used to block fluorescence excitation light. (B) Spectra from the excitation LED (λ = 454nm, bandpass filtered at 460/60nm). A reference (λ = 523nm) LED is used for correcting for changes in optical properties due to the presence of absorptive hemoglobin. The transmission curve for the 514nm longpass filter at detection is shown for reference. (C) Schematic of the combined GCaMP and OIS imaging system. GCaMP excitation and reference LEDs share a collinear optical path using dichroic mirrors. Green (λ = 523nm), yellow (λ = 595nm), and red (λ = 640nm) are used together to determine differential concentration changes of hemoglobin. Detection is done using a cooled EMCCD with an 85mm f/1.4 lens. Adapted from Wright et al. 2017. [85]

During optogenetic imaging, OIS is simultaneously collected using all four LED wavelengths. Optogenetic photostimulation (see Sec. 4.3.2, Fig. 4.6) is provided by a separate high powered laser with wavelength centered at λ=473nm, which is delivered to the cortex by a series of galvanometer scanning mirrors and periscope optics. The laser stimulus is interleaved with the frames of LED illumination to avoid corrupting OIS measurements at the site of stimulation; the laser frames are not used for oximetry. A 473nm notch filter placed between the lens and the camera prevents the laser from saturating the camera during short epochs of photostimulation in the laser frame.

All LEDs and the CCD are synchronized and triggered using Matlab (Mathworks, Natick, MA), via a National Instruments Data Acquisition card (National Instruments, Austin, TX). The field of view is aligned to cover the majority of the convexity of the cerebral cortex with anterior-
posterior coverage from the olfactory bulb to the superior colliculus, resulting in an area of approximately 1cm$^2$ with each pixel being approximately 78 μm x 78 μm.

### 4.2.2 Awake Mouse Imaging Protocol

A significant modification of our traditional mouse imaging protocol was required to accommodate safe and effective awake imaging. First, mice are shaved and the scalp cleaned before a midline incision is made along the top of the head to reflect the scalp, while keeping the skull intact. A Plexiglas window with pre-tapped holes outside of the field-of-view for securing to a mounting bracket is adhered to the top of the skull using dental cement (Fig. 4.2A). A second Plexiglas window is attached to the imaging window via the same pre-tapped holes to protect the windows from being scratched while the mice are in their home cages. For some mice in which electroencephalography (EEG) recordings are desired, pilot holes are drilled into the skull, where small stainless steel self-tapping screws are inserted and affixed with dental cement immediately prior to window placement.

Once recovered from the window installation procedure, typically after 1-2 days, mice are behaviorally acclimated to the awake imaging system over a period of 5 days. On day 1, the acclimation procedure begins by placing unrestrained mice on top of a levitating Styrofoam ball positioned under the imaging camera. Mice are allowed to walk freely on the ball for 10 minutes as a technician guides the ball to prevent the animal from falling off. On days 2-5, an aluminum mounting bracket is secured to the cranial window to restrict head movement, and the animal is placed on the levitating ball (Fig. 4.2B). With head movement restricted, mice are acclimated to the imaging system in 10-minute sessions, 2 times per day. This protocol is repeated daily until the animals exhibit no signs of stress and engage in normal behavior (grooming, whisking, ...
walking) while mounted in the imaging system, at which point awake mouse imaging can commence. The secured mouse is then placed at the focal plane of the camera for imaging.

Figure 4.2. Awake mouse imaging techniques. (A) A Plexiglas window secured to the exposed mouse skull with dental cement has three tapped holes around the exterior, outside of the system’s field of view, to either mount a protective cover slip in between imaging systems, or to mount to the head bracket during imaging. EEG electrode locations are illustrated. (B) Mice are secured to the system using a head bracket attached to the window’s tapped holes and placed on a levitating Styrofoam ball to allow natural movement.

4.3 Applications
Instead of being designed to study relatively slow hemodynamics in anesthetized mice, such as in the previous planar imaging system [15], this new awake imaging system was designed to accommodate observation of a wide range of different contrasts and types of functional brain activity, including calcium dynamics, targeted optogenetic stimulation, metabolism (e.g. FAD consumption), and cerebral blood flow [54], [100]. The remainder of this chapter highlights preliminary studies of two of these applications, calcium dynamics and targeted optogenetic stimulation, as well as selected results from each of these studies. A full report of these studies can be found in their respective peer-reviewed journal articles [85], [86].
4.3.1 Fluorescence Calcium Imaging

While hemodynamic signals are a reasonable report of neuronal activity, faster and more direct measures of neural activity can be achieved by imaging calcium dynamics. Calcium concentration changes due to voltage-gated calcium channels can be imaged and visualized using fluorescent, genetically-encoded calcium indicators [101]. Genetically encoded calcium indicators can map spontaneous network activity at frequencies much higher than the canonical functional connectivity band (0.009-0.08Hz) [21]. This opens up access to phenomena that are not accessible with hemodynamics, such as the delta-band slow oscillation (0.5-4Hz) present in sleep and under anesthesia [102]. Green calcium-modulated protein (GCaMP) [103], a fluorophore generated from a modified green fluorescent protein coupled to a calcium binding domain, enables increased fluorescence in the presence of elevated calcium levels. In a GCaMP6 transgenic mouse model [104], GCaMP expression is widespread in the central nervous system and localized predominantly to excitatory projection neurons [105].

Image processing is carried out using previously outlined techniques, with the addition of a correction scheme for removing any contribution of the vascular component and variance in absorptive hemoglobin concentration from the GCaMP6 fluorescent signal. We implemented a ratiometric correction algorithm (Eq. 4.1) to correct fluorescent emission for any absorption by hemoglobin and deoxyhemoglobin using the reflectance channels at the GCaMP6 emission wavelength (523nm LED).

\[
y(t) = \frac{I_{em}(t)}{I_{ref}(t)} \cdot \frac{I_{0,ref}}{I_{0,em}}
\]  

(4.1)
Here, \( y(t) \) is the final corrected GCaMP6 time series for a given pixel, \( I_{em} \) refers to the detected fluorescent emission intensity, and \( I_{ref} \) describes the measured reflectance changes at the emission wavelength. With this technique, we can concurrently collect both calcium activity and hemodynamics, and uncouple them to process individually and directly compare them within a given mouse.

**Results**

To confirm GCaMP6 fluorescence was present in superficial cortical layers, paraformaldehyde-fixed GCaMP6 brain slices were imaged using both epifluorescence (Fig. 2Ai) and confocal (Fig. 2Aii) microscopy, revealing detectable neuronal somas and their neurites throughout cortical layers I-III and V-VI across the cortex. To evaluate the mapping fidelity of both the GCaMP6 and hemoglobin contrasts, as well as the validity of the ratiometric correction strategy, we used a block design electrical hindpaw stimulation paradigm (5s rest, 4s stimulation at 2Hz, 51s rest; 36 blocks across 7 mice) in mice under ketamine/xylazine anesthesia. Maps of the spatial extent of hindpaw cortex, contralateral to stimulation, for both GCaMP6 and oxygenated hemoglobin concentration (HbO\(_2\)) show well localized responses (Fig. 4.3B). Before ratiometric correction, each electrical pulse response is represented in the raw GCaMP6 signal (Fig. 4.3C, dark green) with a rapid decrease seen after initially rising due to increased absorption (Fig. 4.3C, light green) from increased blood volume and HbO\(_2\) concentration (Fig. 4.3C, red trace). The observed ratio of HbO\(_2\) to deoxygenated hemoglobin (HbR) peak magnitudes (Fig. 4.3C, red and blue traces) is approximately 3:1, as expected from canonical functional responses [19], suggesting that the implemented oximetry calculations sufficiently unmix and estimate HbO\(_2\) and HbR dynamics.
Having validated GCaMP6 as a contrast capable of mapping brain activity in mice comparably to hemoglobin, we examined a potential dependence of GCaMP6 activity on state (anesthesia vs. awake) by evaluating seed-based functional connectivity maps of GCaMP6 in the higher (0.4-4.0 Hz) frequency band which is typically inaccessible when imaging only hemoglobin. Under anesthesia (Fig 4.4A, top row), GCaMP6 functional connectivity shows an anterior-posterior separation, with large regions of high magnitude correlations and anti-correlations across the cortex. In contrast, functional connectivity correlation structure in the awake state (Fig 4.4A, second row) exhibits higher spatial specificity for each network of interest. Difference images between the two states (Fig 4.4A, third row) show that awake maps have finer detail than anesthesia maps, with pixelwise t-statistic maps reinforcing this structure (Fig 4.4A, fourth row). Horizontal line profiles through the center of each of each seed for both awake (red) and anesthetized (blue) FC maps show higher focality (represented as a reduction in ipsilateral and
contralateral full-width at half maximum) in cingulate, motor, somatosensory, retrosplenial, and visual networks in the awake state.

**Figure 4.4.** Functional connectivity mapping between states in GCaMP6 mice. (A) Seed-based functional connectivity maps for seven canonical functional networks in anesthetized and awake GCaMP6 mice (N = 7) at delta-band frequencies. Under anesthesia (top), delta activity (0.4±4.0Hz) drives a strongly correlated/anticorrelated network structure between anterior and posterior brain regions that is not observed in awake animals (second row). This high magnitude correlation feature is preserved in the difference images (third row) and after performing pixelwise t-tests and thresholding the t-statistic maps at a Bonferroni adjusted α = 3.1e-6 (fourth row). Horizontal line profiles through the center of each seed show higher ipsilateral and contralateral focality in the awake (red) FC maps compared to anesthetized (blue) maps (fifth row). Adapted from Wright et al. 2017 [85].

### 4.3.2 Optogenetics
While resting state functional connectivity has provided a great basis for mapping large scale brain networks, its relationship to anatomical connectivity is not well understood. Effective connectivity (EC) provides a measure of the influence that one brain region exerts over another, which can reveal aspects of functional connectivity obscured by network-level large scale synchronization seen in typical resting state functional imaging. The awake mouse imaging system has been developed to allow for optogenetic photostimulation of light-gated neurons in mice expressing channelrhodopsin (ChR2, a light-gated ion channel) under control of a
Thymocyte antigen-1 (Thy1) promoter [106], [107]. Because spatial distribution of ChR2 spans multiple layers of the mouse cortex [108], [109], we can use this technique to map effective connectivity via direct electrical stimulation throughout the mouse cortex.

**Results**

First, to calibrate the dose response curve of ChR2-evoked hemodynamic responses and ensure stability of the mapping procedure, laser stimulation was applied to the forepaw region of the somatosensory cortex in 4 awake animals. This established that the mapping procedure met the following criteria: 1) low power (<1mW) optical stimulation to avoid tissue heating [110], [111]; 2) non-saturating dose-response regime [112]; 3) rapid decay of responses to baseline to enable efficient sequential scanning; and 4) high contrast-to-noise ratio (CNR).

Then, to validate that optogenetic stimulation of the brain generates comparable responses to those that would be evoked in an equivalent task-based paradigm, cortical responses of oxy-, deoxy- and total hemoglobin (HbO₂, HbR, and HbT) are measured following stimulus of the forepaw cortex with fixed optical power (0.5mW) and pulse width (5ms) over an effective spot size of 0.24 mm². Frequency and duration are systematically varied at approximately log-spaced intervals. ChR2-evoked hemodynamic responses are found to monotonically increase as a function of both stimulus frequency (Fig. 4.5A, columns) and stimulus duration (Fig. 4.5A, rows). In greater detail, for stimulus train durations of 3 seconds, ChR2-evoked hemodynamic responses increase in magnitude as a function of increasing stimulus frequency up to 10Hz, then plateau at 20Hz (Fig 4.5B, top row). Interestingly, this response profile is not observed in experiments varying stimulus duration with fixed frequency (Fig. 4.5B, bottom row).
The faster response peaks within 2-3 seconds, after which a slower, high amplitude (nearly twice that of the early response), secondary hemodynamic response emerges with a peak time approximately equal to the stimulus duration. While HbO\textsubscript{2} and HbT generally increase in amplitude with increasing stimulus duration, HbR amplitude notably remains relatively stable.

Having determined that photostimulation of the forepaw cortex produces a comparable result to the response following direct electrical stimulus of the forepaw, this technique can be used to map effective connectivity throughout the cortex, providing a modality for simultaneous observation of the functional and structural architecture of the brain of higher order than those typically accessible with traditional planar mapping of hemodynamics.

### 4.4 Conclusions

The development of an imaging system to accommodate awake mouse imaging and the flexibility to probe more than just traditional hemodynamic-based resting state functional connectivity has greatly expanded our ability to examine the functional and anatomical...
architecture of the mouse brain. Calcium activity reveals functional connectivity over a frequency range (from 0.009 to >4Hz) much higher than the canonical functional connectivity band (0.009 to 0.08Hz). This includes the state-sensitive delta frequency band (0.4 to 4.0Hz), and can therefore be used to assess differences in brain function across awake, anesthetized, and naturally-sleeping states. Additionally, calcium-based spontaneous mapping of functional connectivity is more directly coupled to neural activity than hemoglobin-based methods.

Optogenetic mapping of effective connectivity, on the other hand, allows probing of specific functional connections and the rapid targeting any brain region of interest, not just those corresponding directly to traditional task-based paradigms. Effective connectivity mapping also provides complementary information to resting-state functional connectivity mapping regarding the functional organization of the brain. Both of these techniques further our understanding of the relationships between structural connectivity, functional connectivity, and neural activity, and the awake mouse imaging system will continue to provide new opportunities for investigation of brain function and organization. Further, the recently developed custom-illumination multi-view diffuse optical tomography system for mouse neuroimaging (Ch. 3) can incorporate these techniques for full-volume, 3D imaging of fluorescence and effective connectivity of the brain.
Chapter 5: Functional Deficits in Mice Following Ethanol-induced Apoptosis

5.1 Introduction
The selective regulation of cell death, known as apoptosis, is critically important during brain development; for example, cells needed for locomotion in larvae or tadpoles are ablated as insects and amphibians go through metamorphosis [113]. Although this cell death is vital during brain development, recent work has shown that various classes of common drugs (NMDA antagonists and GABA agonists, collectively referred to as NAGAs) dramatically increase apoptosis in the developing rodent brain beyond normal amounts, potentially causing short and long term damage [114], [115]. NAGAs encompass many commonly used clinical medications and drugs of abuse, including all anesthetics and many sedatives, such as isoflurane [116], propofol [117], ketamine [118], and alcohol [119]. This, combined with recent studies showing a similar effect in neonatal or premature primates [120], [121], has led to increased clinical interest in the potential effects of this increased apoptosis on human neurodevelopment.

Vulnerability to NAGAs occurs during the period of synaptogenesis in brain development, which happens between several days before birth and ~2 weeks after birth in rodents. The equivalent stage in human brain development begins late in the second trimester of pregnancy and continues through the first several years of life [122]. Of additional concern, caffeine, which is commonly administered to premature infants in the clinic to prevent apnea during surgery [123], [124], has been found to exacerbate the increased apoptosis when combined with many clinical medicates in rodents [125], [126]. Consequently, exposure to anesthesia during infancy and through the first few years of life, especially if administered in conjunction with caffeine, could be triggering a similar increase in apoptosis in humans. Some longitudinal studies in humans have found, for
example, a correlation between ADHD diagnosis and anesthesia exposure during infancy [127] and a correlation between lower IQ and anesthesia exposure [128], but establishing a cause and effect relationship of this in humans is prohibitively difficult.

However, we can study longitudinal functional consequences of this increased apoptosis during development in mice, and ultimately provide insight into which anesthetics are safe, and up to what amounts of exposure. Long-term, mouse Diffuse Optical Tomography (chapters 2 and 3) and fluorescence imaging (chapter 4) will combine to be strong candidates to carry out such studies, as they will provide full volumetric brain images completely non-invasively that span the entire hemodynamic response instead of just hemoglobin concentration changes in a 2D slice of the cortex. First, as a preliminary validation study in our planar imaging system, we have looked into ethanol exposure in the young mouse, which corresponds to a mother abusing alcohol during the third trimester of pregnancy. Ethanol is a NAGA that causes similar apoptosis as that provoked by the commonly used clinical anesthetics and sedatives, and therefore will reflect the amount to which our imaging techniques are sensitive to any long-term functional deficits brought by anesthesia-induced increases in apoptosis. This work was performed in collaboration with the labs of Prof. Kevin Noguchi and Prof. Joel Garbow.

5.2 Methods
Mice were exposed to 2.5 mg/g doses of ethanol twice (two hours apart) on postnatal days 4, 6, 8, and 10 – the equivalent of a human fetus being exposed to alcohol throughout the third trimester. Some mice were given 80 mg/kg doses of caffeine, a comparable dose to what might be administered to a neonatal infant during surgery, 15 minutes prior to ethanol injection to see if the increased apoptosis brought on by caffeine translates to long-term effects. Control mice underwent the same procedure but were exposed only to saline instead of ethanol or the
ethanol/caffeine combination. To observe and quantify axonal death following apoptosis, some mice were sacrificed 24-40 hours after the final ethanol exposure. Apoptotic cells can be visualized by activated caspase-3 immunolabeling, which rips apart cellular proteins upon apoptosis and can be stained to detect the deaths of both neurons and axons [129], [130]. Any mice not sacrificed for cell death quantification were allowed to age into full maturity (>30 days) before commencing imaging.

5.2.1 Diffusion Tensor MR Imaging
First, after reaching full maturity, animals were scanned using a 4.7T Agilent/Varian DirectDriveTM small-animal magnetic resonance imaging (MRI) system (Agilent Technologies, Santa Clara, CA) equipped with Magnex/Agilent HD imaging gradient coils (Magnex/Agilent, Oxford, UK), using the system and techniques developed by Garbow, et al [131]. Diffusion tensor imaging (DTI) is used to measure fractional anisotropy throughout the brain, which quantifies the directional dependence of water diffusion [132] and preferentially highlights the white matter tracts that are most significantly affected by apoptosis. This allows a calculation of the volume of specific white matter tracts, such as the corpus callosum, pyramids, and fimbria, in addition to the whole brain volume provided by traditional T2 weighted MRI.

5.2.2 Optical Intrinsic Signal Imaging
One to two weeks after DTI, mice were imaged using the optical intrinsic signal (OIS) planar imaging system. Mice were anesthetized with a ketamine/xylazine cocktail, with resting state data collected for ~30 minutes per mouse, sectioned into individual five minute runs. Image analysis and data processing follows the previously outlined techniques, with changes in reflected light intensity converted to changes in hemoglobin concentration, which can then be used to generate seed-based correlation maps of spontaneous activity for each five minute run.
Correlation maps calculated from each individual run in a given mouse were averaged together to compare functional connectivity (FC) strength in individual mice with their corresponding MR imaging data. To permit averaging across different mice, correlation maps are affine-transformed to a common atlas space based on vascular landmarks in the mouse brain that are visible in the field of view of the OIS system. Average FC was calculated for both the control group (N=4) and ethanol-exposed group (N=7) to test for significant FC deficits between the groups. Only two of the mice exposed to both ethanol and caffeine survived until OIS imaging; thus, the 7 mice averaged together to generate ethanol group maps include these two.

5.3 Results

5.3.1 Apoptotic Cell Spatial Specificity
Widespread cortical apoptosis is found to occur throughout cortical layers II and IV/V. Layer II neurons are known to project intracortically through the corpus callosum (white matter connecting the left and right hemispheres) to the contralateral hemisphere [133], [134] (Fig. 5.1A). Layer V neurons in the motor and somatosensory cortices are known to project through the brain as pyramidal tracts [135], which consists of corticospinal and corticobulbar tracts – white matter connecting the motor cortex to various subcortical brain regions, such as the midbrain, pons, medulla oblongata, and spinal cord (Fig. 5.1B). In each of these cases degeneration is seen qualitatively throughout the pathway that matches axonal tracing following injection of an enhanced green fluorescent protein expressing viral tracer into the motor cortex [136]. Apoptosis is seen to occur both after ethanol exposure (Fig. 5.1C) and caffeine/sevoflurane/midazolam exposure (Fig 5.1D), revealing similar patterns from both alcohol and drugs commonly administered in the clinic. The similar patterns of apoptosis
between alcohol and clinical anesthetics/sedatives supports our use of ethanol as an assay for functional deficits following anesthesia-induced apoptosis.

Figure 5.1. Patterns of degeneration due to alcohol-induced apoptosis. (A) A sagittal brain slice showing axonal tracing from the motor cortex (MC) through the internal capsule (IC) and cerebral peduncles (CP). Image courtesy of the Allen Brain Atlas. (B) A coronal brain slice showing axonal tracing from the MC through the corpus callosum (CC) to the contralateral hemisphere. (C) A sagittal brain slice showing apoptosis along the same pyramidal tracts as shown in A, extending to the thalamus (T), 40 hours after ethanol exposure in the mouse. (D) A coronal brain slice showing apoptosis throughout the MC and across the corpus callosum in a similar pattern as the tracer path shown in B, 32 hours after exposure to caffeine combined with sevoflurane anesthesia and midazolam sedation.

5.3.2 Diffusion Tensor MR Imaging of Brain Volume
The qualitative degeneration shown in Figure 5.1 C and D can be quantified using diffusion tensor MRI. Several weeks after ethanol exposure (once the mice are fully developed, >30 days of age), whole brain volumes are calculated using T2-weighted MRI images through automated segmentation tools, while fractional anisotropy identifies boundaries of white matter tracts of interest to allow calculation of their volumes (Fig. 5.2A-D). We observed significant decreases in whole brain volume and all three white matter tracts of interest: corpus callosum, pyramids, and fimbria (connections between the hippocampus and fornix) (Fig. 5.2E, Table 5.1). Overall, mice exposed to caffeine and ethanol experienced a larger reduction in volume of all four regions than
mice exposed to just ethanol. Mice exposed to ethanol saw their largest reduction occur in pyramid volume, while caffeine + ethanol exposed mice saw the largest reduction in fimbria volume.

Table 5.1 Brain volume % reduction vs. controls following ethanol exposure during infancy.

<table>
<thead>
<tr>
<th></th>
<th>Corpus Callosum</th>
<th>Pyramids</th>
<th>Fimbria</th>
<th>Brain Volume</th>
<th>Average Tracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>21.9%</td>
<td>25.3%</td>
<td>19.5%</td>
<td>11.8%</td>
<td>22.2%</td>
</tr>
<tr>
<td>Ethanol+Caffeine</td>
<td>31.3%</td>
<td>29.0%</td>
<td>32.2%</td>
<td>18.0%</td>
<td>30.8%</td>
</tr>
</tbody>
</table>

Figure 5.2. MRI imaging volumes of mice after ethanol exposure during infancy. (A) A coronal slice of a T2 weighted MR image from a representative mouse. (B) Automated segmentation tools identify the boundary of the brain in each slice, calculating the overall brain volume in each mouse. (C) Coronal slice of a diffusion tensor image of fractional anisotropy in a representative mouse, with white matter tracts revealed more prominently. (D) Automated segmentation tools identify the boundaries of white matter tracts of interest: corpus callosum (red), fimbria (green) and pyramids (blue). (E) Significant decreases in volume are seen for the whole brain and all three white matter tracts of interest in both ethanol exposed mice and caffeine + ethanol mice, as compared to those exposed to just saline.

5.3.3 Optical Imaging of Functional Deficits
One to two weeks after diffusion tensor imaging was performed to quantify anatomical deficits in mice with ethanol-induced apoptosis, we collected optical intrinsic signal imaging data to look for similar deficits in the functional architecture of the brain. After averaging resting state FC maps across each group, visual inspection reveals strong homotopic connectivity in most seed regions across both groups, except for a noted lack of symmetry in the somatosensory seed for
the ethanol-exposed mice (Fig. 5.3A). A two-way ANOVA comparing the bilateral connectivity in each seed location across the two groups reveals a significant decrease in connectivity of the somatosensory cortex (Fig 5.3B). This significance survives a post-hoc Bonferroni correction to account for multiple comparisons.

Figure 5.3. Functional connectivity in adult mice after neonatal ethanol exposure. (A) FC maps of 7 mice exposed to ethanol during infancy (top row) and 4 control mice exposed to saline (bottom row). Pearson-R correlation coefficients are calculated as the temporal correlation between each pixel’s time trace and the time trace at the respective seed location (black dot) in each map. A qualitative lacking in bilateral symmetry can be observed in the somatosensory seed for the ethanol-exposed group. (B) A two way ANOVA comparing the bilateral FC in each seed location across groups reveals a significant decrease in somatosensory FC. (C-F) In 8 mice that had both functional imaging and MR imaging data collected, individual bilateral FC strength in the somatosensory cortex is compared to the four volumes of interest. Correlations are found between SS FC strength and the whole brain volume (C, p<0.005) and corpus callosum volume (D, p<0.05), while trends between pyramid (E) and fimbria (F) volumes were not significant.

For all mice that had both resting state OIS data and MR volumetric data collected (N=8), bilateral functional connectivity of the somatosensory cortex was compared with the normalized volume of the whole brain and the three white matter tracts of interest (Fig. 5.3C-F). Functional
connectivity strength in the somatosensory cortex was found to be highly significantly correlated with whole brain volume (p<0.005) and significantly correlated with corpus callosum volume (p<0.05), despite the relatively low number of animals in the study. While there also existed a trend between functional connectivity strength and the volumes of the pyramids and fimbria, these correlations were not found to be significant.

5.4 Discussion/Conclusion
While the hazards of ethanol exposure, in addition to many other sedatives and anesthesia, during infancy are well understood, optical neuroimaging opens up a new pathway for studying the long-term functional consequences of the subsequent increase in apoptosis. We have shown optical imaging to be sensitive to changes in the functional architecture of the somatosensory cortex, which is of high clinical significance given that most previous techniques quantifying the effects of ethanol or anesthesia exposure during infancy required sacrificing of the animal in question to study individual brain slices. Furthermore, while increased apoptosis only occurs upon anesthesia exposure during infancy, we have shown that the corresponding functional deficits persist into adulthood. We should be able to replicate these findings with larger groups, and in particular we can include a significant number of mice with both ethanol exposure and the ethanol/caffeine combination to quantify whether or not caffeine exacerbates these functional deficits.

Additionally, we have shown that these functional deficits are highly correlated with known volumetric changes in the mouse brain observed with diffusion tensor MRI. This cross-modality significance provides a pair of minimally-invasive techniques that provide information on both functional and anatomical changes following ethanol-induced apoptosis. Having confirmed the sensitivity of optical techniques to these deficits, we can focus on studying these effects
longitudinally in infant mice using Diffuse Optical Tomography, which due to its non-invasiveness and capabilities of producing volumetric functional maps of the brain, might reveal how these functional deficits manifest. This could ultimately be of high clinical relevance, as it could provide information on which specific anesthetics or sedatives cause the greatest deficits, and quantify safe amounts and ages at which the drugs can be administered to infants.
Chapter 6: Conclusion

Diffuse optical tomography as a modality for functional neuroimaging is still in its relative infancy compared to the well-established functional magnetic resonance imaging and positron emission tomography. Although diffuse optical tomography has recently experienced enough technical improvements to achieve sufficiently high resolution and depth localization to be considered for clinical monitoring of brain function in humans, optical methods for mouse neuroimaging have lagged behind due to their being limited to minimally invasive planar techniques. I have demonstrated that diffuse optical tomography, when incorporating structured patterns for source illumination, can be used for non-invasive volumetric functional imaging of the mouse cortex, with recent developments showing promise to image with reasonable resolution throughout the entire volume of the mouse brain.

I have also shown that brain damage caused by anesthesia in infancy causes long-term functional deficits that optical techniques are sensitive to. Furthermore, these deficits are correlated with magnetic resonance imaging of various brain region volumes, providing a cross-modality validation that relates functional and anatomical neurological changes. Additionally, I have aided in the design and assembly of a system that can assess the relationships between functional and anatomical brain structure via the use of optogenetics. This system has also explored new modalities for imaging brain dynamics beyond just hemoglobin, and how brain function is affected by anesthesia, and therefore will be a highly valuable technique for further studies regarding the effects of anesthesia on infant brain development.

Moving forward, structured illumination diffuse optical tomography should allow for highly clinically relevant studies of the mouse brain, in particular the longitudinal observation of functional brain development in the infant mouse and the deviations from normal growth in the
presence of disease. Our recently developed ability to study the differences in brain function between anesthetized and awake mice will permit a more thorough investigation into the effects of anesthesia on the developing brain. Further, expanding the structured illumination diffuse optical tomography system to accommodate calcium fluorescence and optogenetic imaging, a relatively straightforward task given the flexible framework on which it was built, will allow studies of neurovascular coupling throughout the volume of the mouse brain in a wide-range of populations, significantly narrowing the gap between studies of mouse models and their clinical correlates in humans.
References/Bibliography/Works Cited


88


Appendix: System Design/Construction Challenges

A.1 Introduction
The structured illumination diffuse optical tomography (DOT) system for volumetric mouse brain imaging was first theorized with the goal of meeting a number of parameter improvements over previous systems. In particular, we needed high spatial sampling, of ideally ~0.3mm, fast imaging speeds of >2Hz and ideally up to 10Hz, and full volume imaging over a region of ~3cm$^3$. The preliminary proof of concept of the capabilities of this system were founded in simulations of sensitivity and point perturbation reconstructions (see Sec. 3.3.3). The initial design called for four projectors and three charge-coupled device (CCD) cameras symmetrically arranged around the mouse head. Simulations were carried out assuming optics that permit each projector to illuminate 120° of the mouse head, with structured patterns linearly spanning 15 spatial frequencies from 0.04mm$^{-1}$ to 0.6mm$^{-1}$. Reconstructions of 200μm x 200μm x 200μm point perturbations revealed expected resolution and depth localization throughout the full volume of the mouse head. These preliminary studies showed enough validity for funding for this system to be obtained; hence, the initial goal of my research was to design and assemble the system to meet the desired characteristics.

A.2 Structured Illumination DOT System Construction

A.2.1 Camera Selection
The first and most important design decision was the selection of a camera to achieve the desired signal-to-noise ratio (SNR) over the range of wavelengths intending to be used (visible through near-infrared). While very expensive (~$50k) scientific cameras have good sensitivity to all visible and near-infrared wavelengths, a primary goal of this system was to demonstrate that optical tomography can perform comparably to functional magnetic resonance imaging for
considerably cheaper. Therefore, we focused on finding a more affordable camera capable of achieving sufficient SNR for tomographic imaging.

Using the Andor iXon 897 as the gold standard scientific camera for optical imaging systems, as it has already been established as a suitable camera for detecting resting state functional connectivity in mice (Chs. 1, 4, 5), we surveyed several cameras and compared their SNRs across the visible and near-infrared spectrum to that of the iXon. Adding noise to a typical set of our planar imaging data reveals that an SNR of at least ~1.2 is necessary to observe any resting state functional connectivity structure, while an SNR of greater than ~2.5 is necessary to observe clean (i.e. indistinguishable from data with no noise added) functional connectivity maps. This helped to set a lower threshold on a suitable SNR for the structured illumination DOT system.

The signal acquired in an optical imaging system for a given wavelength is traditionally considered to be the product of photon flux incidence (P, the photons per pixel per second), quantum efficiency (Qe, the ability of a sensor to convert detected photons into electrical current at that wavelength), and the exposure time (t). The noise is defined as the square root of the sum of three components: the signal (corresponding to the shot noise, or the statistical variations in photon arrival rate), the dark current (D, in electrons per pixel per second, caused by thermal fluctuations during detection) of the sensor multiplied by the exposure time, and the square of the readout noise (Nr, inherent electron noise in analog-to-digital conversion) [137]. For typical resting state imaging, our true “signal” is the spontaneous fluctuation as a deviation from baseline, which is typically on the order of a 3% change [15], [54]. As a result, the SNR for all subsequent calculations scales the signal by 0.03:
At the typical photon incidence for a standard wide-field optical imaging system (~10mW incident LED power) of ~$10^5$ or $10^6$ photons per pixel per second, the total noise is highly dominated by the shot noise (the first term in the denominator of equation A.1). In this case, the SNR as a function of wavelength will almost identically match the specified quantum efficiency of that wavelength range for the sensor in use (Fig. A.1A, B). However, if we consider lower light levels (~$10^3$ photons per pixel per second), which ideally this tomography system will be capable of imaging as well, as may be required for fluorescence imaging (Ch. 4), the dark current and readout noise begin to affect SNR (Figure A.1C).

Exploring the SNR vs wavelength for three types of sensors, the gold standard expensive CCD (Andor iXon), a cheaper CCD (Basler Aviator avA2300), and a mid-priced sCMOS sensor (Andor Zyla), we see that the sCMOS sensor provides sufficiently high SNR over the full visible spectrum.

\[ SNR = \frac{0.03 * P Q_e t}{\sqrt{P Q_e t + D t + N_r^2}} \]  

(A.1)
and near-infrared spectrum. Additionally, this sensor has enough pixels (5.5 million total) to accommodate flexible binning and cropping, which should allow adjustments of speed and dynamic range to achieve the desired values.

Having identified a sensor that will provide sufficient SNR across the full range of anticipated wavelengths, our camera selection was narrowed down to two similarly priced, commonly used sCMOS cameras which both utilize this sensor: the Andor Zyla and PCO Edge. Additionally, both cameras had two options for communication between camera and computer: CameraLink and USB3.0. While CameraLink generally affords faster frame rates, USB 3.0 is becoming a standard across computer types and frame grabbers (the hardware that converts the digital signal into usable data on the computer), so we elected to sacrifice speed slightly in the interest of system longevity and flexibility.

The primary deciding factor in favor of the Andor Zyla is its flexible readout mode. Because we are monitoring hemodynamics over the entire volume from every region (pixel) simultaneously, we need a camera that reads out all pixels simultaneously. While both cameras implement a feature (called “global shutter”) accommodating this, only the Zyla can achieve this without further sacrificing frame rate. For example, the maximum frame rate that the PCO can achieve at full resolution in global shutter mode is 16 frames per second, while the Zyla can run at 39 frames per second at full resolution.

Although the well depth (maximum electrons detectable per pixel per frame) of the Zyla is significantly less than the Andor iXon (~30,000 electrons compared to 180,000 electrons), the Zyla has a surplus of pixels which can be binned to effectively increase well depth and consequently the dynamic range. With the Zyla sensor cropped to 512x512, it can run at 201
frames per second (see Sec. A.3.3), while the iXon can run at 206 frames per second with 4x4 binning of its 512x512 pixel CCD sensor. Binning the Zyla sensor down an additional 4x4 increases its per-pixel dynamic range to values comparable to the iXon, with the only sacrifice being spatial sampling. Off-camera binning after data collection condenses the data to 32x32 pixels; this makes for a more manageable computational problem (see Secs. A.2.3 and A.3.4) while still preserving the desired spatial sampling. Given the pixel size, these binned “super-pixels” are ~400x400μm each, close to the original goal of 300μm spatial sampling. While this camera may not be sufficient for extremely low-light-level, single-photon type applications, the 4x decrease in cost for just slightly worse dynamic range (or equivalent dynamic range with slightly worse spatial sampling) is a worthy sacrifice.

A.2.2 LED and Wavelength Selection
Camera selection was based off of the “ideal” illumination, with typical estimates for LED power, attenuation, and optical throughput found in the visible spectrum used to estimate typical photon incidence and subsequently used to calculate SNR. However, we needed to make sure the full range of possible wavelengths would provide the necessary signal for this camera before making purchases. This problem can be solved using a concept known as detectivity, which, for a particular sensor and sampling rate, describes the minimum incident power detectable. To calculate detectivity, we start with the sensitivity of the sensor, which describes the number of detected counts per unit energy for a given wavelength:

$$Sensitivity = \left(\frac{QE_{\lambda} \cdot ADC}{E_{\lambda}}\right)$$

(A.2)

Here, $QE_{\lambda}$ is the quantum efficiency of the sensor at that wavelength, $ADC$ is the analog-to-digital conversion rate (in counts per electron), and $E_{\lambda}$ is the energy of a photon of the given
wavelength. This can be converted to a noise-equivalent energy by taking the read noise ($\sigma_{RN}$) of the sensor (in counts) and diving it by the sensitivity:

$$NEE = \left(\frac{\sigma_{RN}}{\text{Sensitivity}}\right)$$

(A.3)

To account for the sampling rate of image collection, this can be converted to a noise-equivalent power, which scales the noise-equivalent energy by the frame rate, and is generally then normalized by the square root of the frame rate ($FR$) to describe the quantity as a function of bandwidth:

$$NEP = (NEE \times \frac{FR}{\sqrt{FR}})$$

(A.4)

Finally, the detectivity is defined as this noise-equivalent power normalized by the area of the sensor ($A$):

$$Detectivity = \left(\frac{NEP}{A}\right)$$

(A.5)

This quantity can then be calculated for a given sensor and choice of sampling rate and sensor area (if cropping). In our case, we can look at the expected power output of the candidate LEDs to make sure that their signal will remain above the detectivity of the camera over the transmission depths that we anticipate imaging. For the off-the-shelf Lightcrafter 4500 projector with built-in LEDs (see Ch. 2), the three included wavelengths are incapable of providing sufficient signal for the Andor Zyla sensor to allow imaging of the full volume of the mouse brain (which requires ~2cm total transmission length), with green and blue mostly only able to probe the cortex (~5mm transmission length, Fig. A.2A).
Figure A.2. Detectivity of Andor Zyla cameras. (A) The Lightcrafter 4500’s three built in LEDs all fall below the detectivity of the camera before the necessary 2cm transmission length for whole volume imaging. (B) Estimates of power output following 2cm of light transmission for the wavelength range of 350-900nm (dotted blue). Wavelengths where the dotted blue line falls below the camera detectivity are not capable of probing the entire mouse brain. (C) Six candidate LED wavelengths with attenuation estimated from true measures of their spectra and incident power, with power falloffs throughout the mouse head volume up to 3cm transmission length. Near-infrared wavelengths show an ability to probe the entire volume of the mouse brain.

To determine wavelengths that will combine to give the desired sensitivity at large (~2cm) depths while maintaining good resolution at shallower depths, we can approximate the intensity of light following a 2cm transmission through the mouse head by assuming a Gaussian LED spectrum to calculate baseline optical properties at all wavelengths from 350 to 900nm (Fig A.2B, dotted blue line). Highlighting the wavelengths of six candidate external LEDs (Mightex systems) shows that the shortest wavelengths will not be able to probe the full volume of the mouse head, but longer (near-infrared) wavelengths will have power remaining significantly above the noise floor of the camera. Looking at the light intensity fall-off for each of these LEDs using their true spectra and incident powers confirms the ability of the near-infrared wavelengths to probe throughout the volume of the brain (up to ~2.5-3cm transmission length) before falling below the noise floor of the Andor Zyla camera (Fig. A.2C).

A.2.3 Computer Selection
Computer selection for the structured illumination system required two main criteria to be met: hardware to accommodate connecting and triggering all of the various components of the system, and hard drive speed and throughput capable of transmitting and spooling the data at the desired
frame rates. At a maximum, the structured light system would incorporate 24 LEDs (four projectors with 6 LEDs each), 4 projectors, 3 cameras, and an electrical stimulus for evoked response experiments. Hence, we needed a data acquisition card capable of controlling and triggering up to 31 independent components, and a computer capable of supporting this card in addition to its other requirements. National Instruments manufactures one card that fulfills these requirements, the 32 analog output channel PCI-6723, which subsequently required a computer with a PCI bus accommodating this somewhat outdated form factor.

To account for this, we assembled a custom control computer from Dell that met the necessary hardware requirements. With the expected cropped field of view, before binning, data collection occurs at 1024x1024 pixels and a maximum of 100 frames per second. With 16-bit imaging, this corresponds to 200MB/sec of data. To accommodate this, we selected an internal PCIe solid state drive for data spooling, which has maximum write speeds of up to 1200 MB/sec, but typically runs closer to 500-600MB/sec. If spooling 2x2 binned data with the same 100Hz frame rate, the system collects ~40MB of data per second. For a standard 5-minute imaging run, this comes to ~12GB of data, for a total of ~80GB of data per mouse. Data are binned further before processing to lessen computation time, but to save raw data, hard drive space can become occupied very quickly. As a result, we selected a computer with hot-swappable hard drive docks, so that regular rapid filling of raw data drives would not require a complete system shutdown for replacement. Instead, data are copied from the PCIe solid state drive to removable hard drives for long-term storage. For the preliminary system (Ch. 2), with just one camera and one projector, the same computer was used for control and spooling. For the multi-view system (Ch. 3), each of the three cameras had its own computer for spooling, with a single separate control computer used.
A.2.4 Optics Selection

The primary challenge in selecting lenses and designing optics for the structured illumination system was in preserving the desired field of view throughout illumination and detection within the confines of the spatial constraints of the system components. For the desired field of view of \(~1\text{cm} \times 1\text{cm}\) (from the top down 2D view of the mouse head/brain), the lenses would have to magnify the \(9\text{mm} \times 7\text{mm}\) digital micromirror device (DMD) chip, which produces the structured light patterns, to at least \(1\text{cm}\) along the smaller dimension to illuminate the full field of view. To preserve symmetry along all sources and detectors, the same lenses were desired for the cameras and projectors. As such, the lenses needed to scale the \(~1\text{cm} \times 1\text{cm}\) field of view to the cropped camera sensor size of \(6.67 \times 6.67\text{mm}\).

![System optical components arrangement](image)

**Figure A.3.** System optical components arrangement. Each projector and camera has an \(85\text{mm}\) focal length lens at a working distance of \(21.3\text{cm}\) (drawn approximately to scale) from the imaging plane, with distances between the lens and the corresponding sensor or digital micromirror chip of \(14.15\text{cm}\). This generates the desired magnifications throughout the imaging system and allows for efficient spatial arrangement of the cameras and projectors.

With the DMD, sensor, and mouse head all being close in size, optics were chosen to utilize close to a “2f” relay, meaning the working distance is approximately twice the focal length of the lens. Given the standard diameter of the large numerical aperture camera lenses in use, and the
sizes of the body of the Andor camera and Lightcrafter 4500 projector light source, the working distance was required to be around a minimum of 20cm (Fig. A.3). To optimize the two light paths and maintain efficient use of space, 85mm focal length lenses were chosen to sit 21cm from the imaging plane, and about 14cm from the sensor and DMD chip, which generates the desired magnifications.

A.2.5 Software
To control the timing of the various components in the structured illumination system, voltage triggers are generated in Matlab and sent to the different devices using the PCI-6723 data acquisition card discussed in section A.2.2. Because the Andor camera and Lightcrafter 4500 projector are both controlled by independent software, their respective settings must be set up and queued before beginning collection. This consists of setting the desired exposure time (which defines the amount of time passing between a frame’s initialization trigger and image readout), number of frames (for allocating necessary space on the hard disk), and binning information on the camera. For the projector, all patterns are created as individual bits in a 24-bit .bmp image in Matlab and stored on the on-board memory. This allows more efficient storage of structured light patterns, as 24 separate patterns (assuming binary patterns where each DMD is either “on” or “off”, as opposed to the use of spatial sine waves in traditional structured illumination) are contained in a single .bmp image and can be triggered more rapidly. The order of patterns to be displayed in the sequence must be loaded from the on board memory before imaging, and is done using the Lightcrafter 4500 software. For the preliminary system, the color of each pattern was assigned in this software as well. For the multi-view system with custom light engine and illumination, a separate series of triggers is required for each LED (Fig. A.4).
Figure A.4. Structured illumination DOT trigger sequences. This sequence is for a hypothetical pattern sequence of 10 spatial frequencies, all six wavelengths per projector, and 120 frames per second collection on the cameras. LEDs are sequentially illuminated while each individual pattern is triggered. Once all 10 of the patterns have been triggered for a given wavelength, the next wavelength is triggered and subsequently illuminated with the same 10 patterns. After all six wavelengths have illuminated all 10 patterns each, the same sequence is carried out for the second projector. All three cameras are triggered at the constant frame rate throughout.

A.3 Multi-view System Construction
While design decisions were made with the ultimate application of multiple detector views and multiple projector illumination angles in mind, the eventual addition of this expansion required solving further design challenges.

A.3.1 Custom Light Engine
In order to accommodate a range of LEDs and wavelengths and preserve flexibility to exchange LEDs in the future if necessary, we designed a light engine that would sit away from the system and can be adjusted independently from the cameras and projectors. Six LEDs are combined with appropriate dichroic lenses as a part of this light engine, with a liquid light guide transporting the output into the system for DMD illumination (see Fig. 3.1). Although the Mightex LEDs in use are manufactured with a collimating lens (Fig. A.5, C1), because the LED
die (the chip that produces the light output) has some breadth, the collimated light emitted from the LED still experiences some divergence (Fig A.5, $\theta_1$).

Figure A.5. Light engine ray diagram sketch. Light from an LED die radiates outward before collimation by the manufactured collimating lens (CL). Despite collimation, the breadth of the LED die causes continued divergence ($\theta_1$). To focus this large diameter ($h_1$) beam down to the size of the liquid light guide ($h_2$), a pair of lenses (TL1 and TL2) is used and chosen such that the ratio of their focal lengths is equal to the desired magnification. At the point of focus, the divergence increases significantly ($\theta_2$) due to the conservation of the optical invariant. Optical ray tracing courtesy of Davidson College Physlets (http://webphysics.davidson.edu/Applets/Applets.html).

The diameter of the liquid light guide, where light from the LEDs is focused in order to transport light to the projector (see Fig. 3.1B), is much smaller (~1cm, $h_2$) than the diameter of the of the LED collimating lens (~4cm, $h_1$). To shrink this spot size down, we can use a pair of lenses in an optical relay similar to a telescope (Fig. A.5, TL1 and TL2), with their focal lengths ($f_2$ and $f_3$) chosen such that their ratio equals the desired magnification. However, focusing a diverging beam to a smaller area causes the divergence to occur much more quickly (Fig A.5, $\theta_2$), due to the conservation of a quantity known as the optical invariant [138], which states that the product of an optical component’s diameter and its divergence angle is conserved through a system. For the optical components and parameters in Figure A.5, $h_1 \cdot \theta_1 = h_2 \cdot \theta_2$. Because of this rapid
transmission coefficient through each optical element independently. This provided an estimate of the idealized power throughput for each wavelength (in the absence of any power loss besides normal attenuation through the various glass elements), which could then be compared to the true power output of the light engine to see how much power loss is occurring due to imperfect alignment, with subsequent

Figure A.6. Light engine lens selections and locations. Zemax models light ray traces precisely for specific selections of lenses. Given the relative sizes and locations of the LED die, Mightex collimating lens, liquid light guide focusing lens, and liquid light guide tip, we explored a range of possibilities for singlet lens location and focal length to find the option that minimizes light loss for each of the six wavelengths and their respective paths through the light engine.

In order to determine a suitable solution of lens sizes, locations, and focal lengths for the light engine, we utilized a common optical engineering software (Zemax, Fig. A.6). Given the fixed locations and sizes of the LED dies, Mightex collimating lens (CL in Fig. A.5), liquid light guide focusing lens (TL2 in Fig. A.5), and liquid light guide, we selected singlet lenses (TL1 in Fig. A.5) that would preserve as much of the light output from the collimating lens as possible. We found that either a 150mm or 250mm focal length singlet lens was sufficient for containing the majority of the light for the shorter and longer light engine path lengths, respectively. To minimize power loss through the light engine, we measured the transmission coefficient through each optical element independently. This provided an estimate of the idealized power throughput for each wavelength (in the absence of any power loss besides normal attenuation through the various glass elements), which could then be compared to the true power output of the light engine to see how much power loss is occurring due to imperfect alignment, with subsequent
adjustments to optical elements to maximize the power output as much as possible (see Table A.1, columns 1-4).

### A.3.2 Custom Projector

With the power output from the light engine sufficiently optimized, we then needed to use the output of the liquid light guide to illuminate the digital micromirror device (DMD) array (see Fig. 3.1D). I disassembled the commercial Lightcrafter 4500 and used just its crucial components (Fig. A.7A, B), in addition to other optical components chosen to focus the light output down to the DMD chip location. Precise angles of illumination incidence and DMD output with respect to the total internal reflection (TIR) prism are necessary for proper alignment and power throughput. To achieve this, I designed and 3D printed a frame for mounting all of the necessary optical components by measuring the geometry of the commercial projector and duplicating the crucial aspects of its frame as closely as possible (Fig. A.7C).

![Figure A.7. Custom projector. (A) The total internal reflection (TIR) prism, which allows light to pass straight through along one dimension to illuminate the digital micromirror device (DMD) array, and reflects returning light at a 90° angle to send the structured patterns out of the projector. (B) The DMD chip affixed to its electronic flex cable for connection to the Lightcrafter 4500 electronics board. The chip is also affixed to a heat sink for cooling, with black matte tape surrounding it to prevent unwanted excess light from reflecting out of the projector. (C) A 3D printed frame modeled after the crucial components of the Lightcrafter 4500. A lens is affixed at the liquid light guide (LLG) input location to focus light through the TIR prism and onto the DMD chip. Four elevated posts allow stable mounting of the electronics.](image-url)
To verify that our light engine will produce the necessary incident power from each LED to achieve the depth sensitivity discussed in section A.2.2, we measured the power output following transmission through the projector to ensure suitable power (>1 mW) at the imaging plane. Alignment of custom projector components could then be modified slightly until sufficient power was achieved for all wavelengths at the imaging plane (Table A.1, column 5).

**Table A.1 Light engine and custom projector power throughput.**

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Initial LED power</th>
<th>Ideal Light Engine Output</th>
<th>Measured Light Engine Output</th>
<th>Incident Power at Image Plane</th>
</tr>
</thead>
<tbody>
<tr>
<td>470 nm</td>
<td>417 mW</td>
<td>279 mW</td>
<td>160 mW</td>
<td>15 mW</td>
</tr>
<tr>
<td>530 nm</td>
<td>217 mW</td>
<td>64 mW</td>
<td>26 mW</td>
<td>3 mW</td>
</tr>
<tr>
<td>625 nm</td>
<td>165 mW</td>
<td>55 mW</td>
<td>29 mW</td>
<td>5 mW</td>
</tr>
<tr>
<td>680 nm</td>
<td>44 mW</td>
<td>27 mW</td>
<td>26 mW</td>
<td>1 mW</td>
</tr>
<tr>
<td>740 nm</td>
<td>547 mW</td>
<td>79 mW</td>
<td>33 mW</td>
<td>3 mW</td>
</tr>
<tr>
<td>850 nm</td>
<td>292 mW</td>
<td>120 mW</td>
<td>82 mW</td>
<td>3 mW</td>
</tr>
</tbody>
</table>

**A.3.3 System Speed**

As discussed in section 3.4.1, the full range of illumination patterns and wavelengths at wide field-of-view, with ~50% overlap between each pair of camera’s pixels, is limited to imaging at ~0.33 Hz. However, the fields of view of the individual cameras can be further cropped to increase speed at the cost of fewer overlapping measurements. The sCMOS camera in use sequentially reads out frames along its columns of pixels (Fig. A.8A, yellow); cropping along this dimension allows each frame to be read out more quickly, as fewer columns of pixels are in use. Therefore, rectangular fields of view can be read out more quickly than square ones. Using the 3D printed action figure, we explored a number of possible fields of view that allow for faster
fame rates, which would ultimately permit the necessary faster full tomography frame rates should large numbers of wavelengths or illumination patterns be desired.

Figure A.8. Field of view vs. frame rate. (A) The standard field of view and spatial sampling of the system permits camera collection at 100 frames per second over 13 x 13 mm. The sCMOS sensor reads out one column at a time, highlighted in yellow. (B) Cropping the sensor by ~20% generates a field of view equal to that in previous planar imaging systems (10 mm x 10 mm), with an increase in maximum frame rate to 132 frames per second. (C) Given the column-wise readout of each frame, cropping the horizontal dimension by a factor of two will keep the vertical dimension constant and yields a 50% increase in maximum frame rate.

The standard field of view has a maximum frame rate of 100 frames per second (Fig. A.8A). Cropping this field by ~20% while keeping it symmetric yields a ~1 cm x 1 cm area (matching that which is used in typical planar imaging systems – see Chs. 1, 4, and 5), with the maximum frame rate increased to 132 frames per second (Fig. A.8B). However, given the column-wise readout of the sCMOS sensor, the vertical dimension can be kept constant while the horizontal dimension can be cropped for a linear increase in frame rate. For example, cropping the horizontal dimension by a factor of two increases the maximum frame rate to 200 frames per second (Fig. A.8C). Performing this same crop on each camera would remove the majority of overlapping measurements in exchange for an overall factor of 2 increase in system speed.

Further, different choices for camera lenses on the projectors and cameras could permit faster frame rates while preserving overlapping measurements at the expense of dense spatial sampling.
A.3.4 Computational Challenges
The pattern sequences in use generally yield 36 patterns (sources) and ~3,000 detectors over the head across all three cameras after all binning and masking is complete, for a total of ~100,000 measurements per wavelength. The overall voxel grid has dimensions of 48x48x48, of which approximately 50% are generally identified as brain, which comes to ~50,000 voxels to be reconstructed per wavelength per run. The sensitivity matrix then has dimensions of ~100,000 x 50,000, which with double precision accuracy corresponds to a single matrix of >32GB data size. Inverting the sensitivity matrix demands more than twice that amount of computational space, as the full information of the matrix is required in calculating its inverse. Consequently, we required a computer with sufficient RAM to accommodate this calculation, in addition to efficient means of communication and data access across collection and processing computers. Our solution has been to generate Green’s functions first and independently for each mouse. Then, using a dedicated computational server with large amounts of memory, we create and invert the sensitivity matrices based on the appropriate sums of the Green’s functions, following the methods of Section 3.2.2. Even with the computational power afforded by this distribution of processing across computers the full data processing for a single mouse (~35 minutes of data), if calculating all Green’s functions and the sensitivity matrix for this mouse, requires ~4 hours of computation time, making anything close to real-time feedback of in vivo imaging data difficult to achieve. As a result, the simulations explored in Section 3.3 are very valuable for optimizing system and reconstruction parameters while avoiding the massive amount of computation time required for full sets of in vivo data.