A Single Board Computer Implementation of Mouse Dual Brain Mesoscale Imaging and Radio Frequency Identification

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Committee Page

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Abstract

The power of social interaction and touch is undisputed across the animal kingdom. To evaluate neuro-correlates of social behaviour, recent studies have focused both on system level human studies to mechanistic studies within animal models. In animals, mechanistic studies have largely focused on social interactions between rodents and this work indicates a fundamental role of the rodent vibrissal system in transmitting social signals between animals. While considerable work has been done in both human and animal models, correlations between subjects over high sampling rates and relatively large expanses of the cortex have not been done. Here, we employ mouse mesoscale GCaMP imaging to establish how calcium activity is correlated when two mice engage in social touch-like behaviour. The two mice begin at a distance where social touch is not possible, then they are transiently brought close to each other in order to allow them to interact. Afterwards, the mice are moved back to their initial separated state. When the mice are in the together state we observe that the mice engage in bouts of mutual whisking resulting in simultaneous barrel cortex activity. Additionally, we find that brain-wide calcium signals at a frequency band of 0.01-0.1Hz become synchronized when the mice are together. We also developed a cost effective radio-frequency based system for mouse identification. We describe a simple protocol to tag and identify mice using glass-encapsulated tags, which were injected sub-cutaneously after brief anesthesia. Python software provides a flexible, cross-platform solution for interfacing with the tag reader, which is capable of reading the tags at a distance of $30.2 \pm 2.4\, \text{mm}$. The system facilitates automated behavioural experiments that require animal identification [Murphy et al., 2016, Silasi et al., 2017, Woodard et al., 2017]. The radio frequency identification (RFID) system could be
applied to further automate social touch studies.
Lay Summary

We have designed and built a brain imaging system that allows us to see the activity of the brain as two mice are brought close to one another to interact. We observed that when the mice are brought to a distance of 12mm from one another, they whisk simultaneously at each other, which results in a similar pattern of activity in both mice. In addition to the mutual whisking, we found that there are brain-wide signals that become synchronized when the mice are at the interacting distance. We propose that this synchrony may be the social interaction signal of the cortex.

We also developed a cost-effective system to automatically identify mice, the mice are implanted with glass RFID tag that contains a unique ID. The glass tags are implanted at the nape of their neck and can be read wirelessly. This setup has been shown to be useful in automated behavioural experiments [Murphy et al., 2016, Silasi et al., 2017, Woodard et al., 2017].
Preface

I wrote chapters 1, 2, 3, and 5 and am responsible for all hardware, software and figures. The digital renders were drawn by my brother Luis Bolanos, and his help was instrumental in making the figures easy to digest by the readers. Chapter 4 consists of a published manuscript by me, Jeffrey LeDue and Timothy Murphy in the Journal of Neuroscience Methods [Bolaños et al., 2017]. Dr. Timothy H. Murphy supervised the project and provided financial support.

Chapters 2, 3 are based of an idea that came about at the 2017 Neurophotonics Summer School. I designed and built all the software and hardware outlined in chapters 2 and 4, with help from Jeff Ledue and Luis Bolanos. I conducted the experiments outlined in chapter 3. The experiments related to differences in cage-mates and non-cage-mates from \( A.1 \) were performed by Matilde Balbi, Luis Bolanos and I, then I analyzed the data. Extremely useful comments and advice were given to me by (in alphabetical order): Alex McGirr, Allen W. Chan, Dongsheng Xiao, Jack Waters, Jamie Boyd, Jeffrey LeDue, Matilde Balbi, Matthieu Vanni. I am eternally grateful for their kindness and support, I am going to miss everyone.
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Glossary

**BOLD** blood-oxygen-level dependent signal

**CIHR** Canadian Institutes of Health Research

**CMOS** complementary metal-oxide-semiconductor

**CSI** camera serial interface

**DTI** diffusion tensor imaging

**FPS** frames per second

**GPIO** general-purpose input/output

**GTM** gradient triggered map

**LED** light emitting diode

**RAM** random access memory

**RFID** radio frequency identification

**ROI** region of interest

**SBC** single board computer

**SNR** signal to noise ratio

**VSD** voltage sensitive dye
WFOM  wide field optical mapping
The past six years have been incredible, and very rewarding. I have learned so many new things, went to many conferences, presented my work in Canada, the U.S. and even in Japan. None of those feats would have been possible without the support and encouragement of my supervisor Dr. Timothy Murphy. I believe that I have grown a lot as a future scientist, and it is in great part, thanks to him.

Thank you to my committee, Dr. Catharine Rankin and Dr. Michael Gordon. It was thanks to their kind words and amazing feedback that I began to think the project I was working on was really interesting.

I also wish to thank the Canadian Institutes of Health Research (CIHR) for their financial support through the Canada Graduate Scholarships-Master’s Program scholarship.

Thank you to Jeffrey LeDue, he was incredibly supportive of my endeavours, and honestly, when things seemed bleak he would show me where to look for the light. I am incredibly in debt to him, and I wish I can see him again in the future. All those conversations that we would have at lunch time were so interesting and I would look forward to them everyday.

Thank you so much to my dear brother Luis, it was especially fun having you at the lab, and thanks to him, my figures look amazing. I believe that they easily convey how we built all of our setups. I hope we get to play Super Smash Bros soon.

Thank you to all the members of the Murphy Lab: Allen, Matilde, Matthieu, Dongsheng, Jamie, Alex, Cat, Pumin and Cindy, everyone played an important role during my years at Tim’s lab. Whether it was random conversations about nothing related to neuroscience, impromptu guitar
lessons, playing with fire, it was all very important to me.

Thank you to my family, my mom, my dad and my sister. My parents’ sacrifice of moving to Canada was instrumental in making me the man I am today. Without their support there is no chance I would be were I am now.

Finally, I would like to thank my girlfriend Victoria for always being there for me when things were hard at the lab, for her love and support of my aspirations, and for figuring out the best methods to motivate me. Coming home and seeing you instantly relaxes me after a stressful day at the lab. Let us keep the adventures and memories coming through my PhD!! :)
Chapter 1

Introduction

Mesoscopic Imaging

The principal goal of neuroscience is to understand how the brain is capable of the extraordinary things animals do; which range from processing the primary sensory information to integrating this information in a multitude of contexts, motor planning, recognition of food and even social interactions. At its core the structure of the brain is known to be a collection of diverse types of neurons and supporting cells that, according to a paper published in 2014, form a complex set of four major network systems; motor, sensory, behavioural states and cognitive states [Oh et al., 2014]. Each of these systems are comprised of specific groups of neurons, and they form intricate connections. The specific connectivity of the neurons are thought to be one of the main contributors to the set of complex behaviours in animals [Seung, 2012]. In recent decades there has been a considerable amount of work to try and decipher the connectome of the brain in several species [Oh et al., 2014, Sporns et al., 2005, Takemura, 2014, White et al., 1986]. Connectome refers to the wiring pattern of the brain, and this description can be done at a structural or functional level. Structural connectivity describes the existence of anatomical tracts connecting two brain regions [Bullmore and Sporns, 2009]. On the other hand, functional connectivity refers to the causal dependance of specific activity patterns in different regions of the brain [Bullmore and Sporns, 2009].
Decoding the useful information behind the patterns of neuronal firing is yet another goal of modern neuroscience. Much has been done describing the morphological, and physiological properties of neurons [Chung and Deisseroth, 2013, Ragan et al., 2012], and there is even a large database of such data published by the Allen Institute for Brain Science all [2017]. One thing is for sure: even for a mouse brain, the complexity and diversity of both the connectome and neuronal activity is immense. For that reason scientists have had the need to study the brain at multiple scales; macro-, meso- and microscale. At the microscale we describe how individual synapses are connected to one another and the electrical activity at the level of individual action potentials, for example, patch clamp experiments on brain slices and reconstructing the connectivity with electron microscopy [Oh et al., 2014]. But describing the brain at this scale is currently infeasible for large brains, due to the large amount of data generated Bock et al. [2011], Briggman et al. [2011], Chklovskii et al. [2010], Helmstaedter et al. [2013], Takemura et al. [2013]. On the other side of the spectrum one can map large scale structural connections utilizing diffusion tensor imaging (DTI) [Greicius et al., 2009], or describe functional connectivity using a slow proxy for neuronal activity, the blood-oxygen-level dependent signal (BOLD) signal [Biswal et al., 1995]. However, this is very far from cellular resolution as the size of the individual voxels is typically no smaller than 1 mm$^3$. To put that in perspective, there are about 92,000 neurons in 1 mm$^3$ of mouse neo-cortical tissue [Schütz and Palm, 1989]. A middle ground is the mesoscale, at this scale it is possible to describe local and distant structural connections between areas of the brain by labelling specific neurons with tracer proteins allowing to map their inputs and outputs [Oh et al., 2014]. Mesoscopic functional imaging is also widely used (also referred to as wide field optical mapping (WFOM)), at this scale both local and global activity changes can be recorded. This makes it suitable for animal models with smaller brain sizes (mice, rats, marmosets, etc). Mesoscopic functional imaging is usually done by making use of a diverse selection of proteins and dyes with varying properties. Some of these molecules can measure voltage changes directly (Butterfly, voltage sensitive dye (VSD)) or a proxy of it, such as intracellular calcium (GCaMP) or extracellular glutamate (iGluSnFR) [Akemann et al., 2012, Chan et al., 2015b, Chen et al., 2013b, McGirr et al., 2017, Mohajerani et al., 2013]. Each of
these sensors have different temporal resolution and they each have advantages and disadvantages, usually in signal to noise ratio (SNR) and how accurate of a proxy it is for the brain’s true electrical activity. For instance the voltage sensors have really fast temporal dynamics but have poor SNR, and this means that imaging with these sensors will require a camera with low noise [Ma et al., 2016a]. On the other hand a sensor like GCaMP has a great SNR, but has slow temporal dynamics with decay constants of above 1 second and is measuring the \( \text{Ca}^{2+} \) inside neurons, which is not brain activity directly [Chen et al., 2013b, Madison et al., 2015]. Nevertheless, GCaMP has been adopted widely mainly due to its great SNR and wide selection of transgenic and viral vectors that allow scientists to express the sensor on specific neurons [Burgess et al., 2017, Dana et al., 2014, Dipoppa et al., 2016, Madison et al., 2015, Pakan et al., 2018]. In this thesis, all the experiments were performed on transgenic mice that expressed GCaMP6s under the Thy1 promoter, which is mainly expressed in excitatory neurons from layers 2/3 [Dana et al., 2014]. In particular we used the GP4.3 line that expresses densely in the cortex, which is the area we imaged.

**Chronic Window Surgery and Mesoscale Imaging in Mice**

In the previous section we described the various scales at which connectivity analysis and *in vivo* imaging has been done in a number of animal models. In particular, we focused on mouse mesoscale imaging as it is the main focus of the research done in this thesis. We presented the idea that imaging at the mesoscopic scale allows us to map activity from both global and local activity. Previous surgical techniques for mesoscopic imaging necessitated partial or full removal of the skull due to the fact that VSDs needed to be applied to the brain tissue prior to being able to image [Holtmaat et al., 2009, Mohajerani et al., 2013]. In addition to the open skull, the dyes themselves have been shown to be phototoxic to the underlying brain, meaning the experiments had to be done in acute and less naturalistic setting [Lin and Schnitzer, 2016, Peterka et al., 2011]. Additionally, the cranial windows that were initially applied to these studies [Holtmaat et al., 2009] were limited in their application to mesoscopic imaging due to the relatively small field of view. Thus, the need for a non-invasive technique that takes advantage of the new generation of activity
indicators without compromising tissue viability while expanding the field of view size. Out of this need a protocol was developed in 2014 that describes a procedure for creating chronic trans-cranial windows on intact bone [Silasi et al., 2016]. Briefly a very similar procedure from this protocol is outlined here; however we utilized a titanium bar instead of the #4 – 40 set screw (See figure 1.1a,c)

**Surgical Procedures**

On a sterilized field, mice are anaesthetized with isoflurane, then they are mounted on a stereotactic frame while making sure their skulls are level with the surface. To prevent eye dryness the eyes are treated with lubricant, their rectal temperature is maintained at a constant 37°C. The mice are given a subcutaneous injection of atropine, glucose, and buprenorphine and their scalp is injected with lidocaine. The fur of the mice is removed exposing the scalp, and the skin is scrubbed three times with a solution of 0.1% betadine and 70% ethanol. To ensure the mice are still under anaesthesia their respiration rate and toe pinch response were checked every 10-15min.
The coverslips are cut with a diamond pen to the dimensions of the final cranial window (See figure 1.1b). Then using a scalpel, a skin flap the size of both hemispheres were cut and removed. Sterile cotton tips are then used to remove any connective tissue or fascia to ensure the surface of their skulls are clean and free of debris. The titanium bars were placed 4mm posterior to bregma with respect to the top edge of the bar, on top of lambda. A single drop of ethyl cyanoacrylate was placed to glue the bars to the skull. Clear dental cement (C&B Metabond) is prepared in order to firmly glue the bars to the skull. Once the initial position of the bars are verified a layer of the dental cement is applied to the side of the bar. The bar is held in place until the dental cement is partially dry (1-2min). With the bar fully glued, a layer of the dental cement is applied directly to the skull. The cut windows are then placed on the skull before the applied dental cement dries. It is important to ensure no air bubbles form while placing the windows because they can negatively affect imaging quality in that area. Additional dental cement is applied around the edge of the windows sealing off the skulls from the air. After the dental cement is fully dry and solid the mice receive a second injection of saline and glucose and they are allowed to recover in their home-cage with a heating lamp. The mice are able to be used for experiments 7 days after the surgery have passed.

Cortical Mesoscopic Imaging in Mice

The aforementioned preparation gives us access to the entire dorsal surface of the mouse cortex which contains several known cortical areas as shown in figure 1.2a, which is a map with areas extracted from the Allen Institute Brain Explorer. Chronic imaging with this preparation has been previously shown to give useful data over multiple months with a spatial resolution capable of resolving intra-hemispheric and trans-hemispheric cortical dynamics [Silasi et al., 2016, Vanni and Murphy, 2014]. These cortical dynamics have also been used in defining functional modules that reflect the connectivity of those regions, and the patterns of activity produced by artificially stimulating those areas reflect the patterns of activity seen in spontaneous activity in awake and anesthetized mice [Mohajerani et al., 2013, Vanni and Murphy, 2014]; Furthermore, cortical os-
Figure 1.2: Brain areas at the mesoscopic scale for the mouse brain and image of the raw data at this scale.

cellations at a wide range of frequencies can also be imaged, and reflect the state of the animal [Chan and Murphy, 2017, Chan et al., 2015a].

The mice we utilized in our experiments expressed the slow variant of GCaMP6 in their cortical excitatory neurons of layers 2/3; we specifically used the Thy1-GCaMP6s 4.3 line from Janelia [Dana et al., 2014]. With this transgenic line we can observe high levels of fluorescence in both hemispheres (See figure 1.2b).

Social Behaviour in Rodents

Mice use their highly sensitive vibrissae (whiskers) to locate objects [O’Connor et al., 2010], discriminate textures [Chen et al., 2013a], and interact with other mice [Brecht and Freiwald, 2012, Grant and Mackintosh, 1963]. Whiskers can be subdivided into two types; the micro and macrovibrissae [Deschênes et al., 2012]. It is the longer macrovibrissae which are closely linked with sniffing that are swept forward (protracted) to provide tactile feedback [Deschênes et al., 2012] during face to face social interactions [Ebbesen et al., 2017].
Mice, like other rodents, display a multitude of social behaviours, most of which can be classified into four categories: attending, approaching, investigating and nosing [Grant and Mackintosh, 1963]. Common assessments of these social behaviours include a 3 chamber apparatus used to measure the degree to which a mouse prefers to interact with a novel stranger mouse rather than a novel object [Crawley, 2007]. During this test the mice are able to engage in social facial touch, although one mouse is segregated from the other by a loose wire mesh, limiting tactile interaction with macrovibrissae. Freely moving animals engage in many social behaviours which can be assessed from video and/or depth sensors and analyzed by human observers or machine learning approaches [Hong et al., 2015]. Commonly quantified behaviours include nose-to-nose contact, nose-to-ano-genital contact, mounting, and attack/escape behaviours.

Recently, head-fixed rats have been used to try to elucidate the cortical signature of facial social interaction using electrophysiological read-out with micro/nanostimulation and pharmacological interventions [Bobrov et al., 2014, Ebbesen et al., 2017, Lenschow and Brecht, 2015]; however, all these studies were restricted to barrel cortex recordings. As an example, in 2015 a study was done exploring the cortical activity of the barrel cortex of a rat that was engaging in a social touch experiment [Lenschow and Brecht, 2015]. Briefly, they found that social touch was correlated with large membrane potential changes and depolarizations that were locked to the rat’s whisking. Mice have been described to engage in a similar behaviour wherein the noses of two mice come in close contact and their vibrissae get directed towards each other [Grant and Mackintosh, 1963]; however, the cortical signature of this behaviour remains unknown in mice, additionally whether or not other parts of the cortex are activated during this behaviour also remain unknown. Thus, one of the principal goals of this thesis is to develop a mesoscopic imaging system that is capable of imaging a large area of cortical activity while two mice engage in social touch like behaviour.

Another important aspect of animal research, especially social behaviour research, requires the precise identification of the subjects due to the nature of the experiments. For example, studies have been conducted to track the interactions of mice in an automated manner by making use of radio frequency identification (RFID) based identification [Howerton et al., 2012, Kritzler et al.,]
A cost-effective method that can be easily implemented for all experiments would greatly reduce human errors and give you an unambiguous and unique identification code for each animal. Therefore, the second principal goal of this thesis is to present a cost-effective, robust and scalable method to identify mice via RFID.

**Research Aims**

To complete our first goal, we developed a paradigm in which two Thy1-GCaMP6s 4.3 mice are head-fixed face to face at a variable distance between 160mm and 12mm such that they can engage in a mutual whisking behaviour; we record cortical calcium activity at the mesoscale in the entire dorsal cortex. We use correlation within and between mice to look at changes in functional connectivity while the mice are brought to a distance that lets them interact. Between mice we observe synchrony of cortical dynamics and identify two phenomena in which high inter-cortical correlations are observed. The first is the mice engage in simultaneous bouts of whisking between each other, which results in synchronized activity in the cortical region corresponding to the macrovibrissae. The second phenomenon we observe is the synchronization of brain-wide signals across the mice when they are together at a specific frequency band (0.01 – 0.1Hz).

To complete our second goal we developed a flexible open-source system to automatically detect and identify mice. Our goal is for the system to be robust over a long period of time, easy to extend over to an automated experimental setup [Murphy et al., 2016, Silasi et al., 2017, Woodard et al., 2017]. The surgery to implant the RFID tags should be relatively non-invasive and quick to complete. Additionally, the code should be cross-platform and easily modifiable to the experimenter’s research pipeline.

To summarize, we have two separate principal goals. The first goal involves the mesoscopic dual imaging system; hardware and software description, along with the results from a set of experiments wherein two mice were allowed to transiently interact compared to two mice who were not allowed to interact. The second principal goal involves the cost-effective RFID based tagging system for mice. Thus, we can split these two goals into three aims that are each described
in the next three chapters of the thesis:

- **Aim 1:** Develop a robust and scalable system for rodent identification based on radio frequency glass tags.

- **Aim 2:** Construct the hardware and the software of an apparatus capable of imaging two mice at the same time while being able to move the mice in and out of their whisker interacting zones.

- **Aim 3:** Perform sets of experiments where you either keep the mice separate or bring them together and then explore any differences in their cortical calcium dynamics.

Aim 1 is covered in Chapter 4 of the thesis and the work outlined in that chapter has been previously published in the Journal of Neuroscience Methods |Bolaños et al. [2017]. Aim 2 deals with an overview of the dual imaging rig, the hardware, the software, and its capabilities and is covered in Chapter 2. Finally, aim 3 deals with the set of experiments we performed in GCaMP6s mice and the results we obtained from the calcium imaging and behaviour data, which is explained in Chapter 3.
Chapter 2

Dual Mice Mesoscopic Imaging Methods

Introduction

In this chapter we describe the hardware of our dual mouse imaging system (See figure 2.2). The system allows for two GCaMP6s expressing mice [Dana et al., 2014] to be imaged simultaneously with variable distance of 12mm and 160mm between their snouts, allowing the mice to engage in a social touch-like behaviour, similar to the one rats exhibit [Bobrov et al., 2014]. Furthermore, we capture high-speed behavioural video of the mice when they are brought to interacting distance. We will describe the lighting system used to image the activity, the stage that keeps the mice fixed, the camera and the settings used as well as a short description of the software that deals with the camera synchronization and data collection and analysis.

Liquid Light Guide Illumination

We image the brain in both reflectance and epifluorescence modes using three distinct light emitting diode (LED)s, each for a different modality (See figure 2.1). We used a short wavelength blue LED with a peak wavelength at 448\(nm\), a longer wavelength blue LED with a peak wavelength at 470\(nm\), and finally a red LED with a peak wavelength at 627\(nm\). The short blue measures blue reflectance signal which reflects changes in blood volume, and we have previously used this wavelength in an attempt to remove blood volume changes to the GCaMP signal [Xiao et al., 2017]. The longer blue
is used to excite GCaMP6s and thus we image the green epifluorescence from the protein. Lastly, we use the red reflected light signal from illumination to measure a different band of the blood volume changes. For further details on the blood volume correction rationale and description see the next chapter.

The light from the three LEDs gets collimated with a 25.4mm biconvex lens, and each of the beams are passed through a separate bandpass filter. The short blue is filtered at a band of $440 \pm 5\text{nm}$, the longer blue with band at $480 \pm 15\text{nm}$, and the red light is filtered at a band of $635 \pm 5\text{nm}$. The reason the lights must be filtered is to avoid cross-talk between the three camera color channels. LEDs tend to have a wide spectrum, for instance the $470\text{nm}$ LED has a wide enough spectrum that it emits green photons, which would bleed into the camera’s green channel. Once the three light beams are collimated and filtered, they combined into a single beam via two dichroic mirrors. Dichroic mirrors are capable of letting through light of certain wavelengths and reflecting light of different wavelengths. In our case we mix the blue lights into the beam path of the red light, so our dichroics reflect blues but lets through anything above greens. Finally, the combined light beam is then focused into the liquid light guide using a 25.4mm biconvex lens. The other end of the liquid light guide can then be placed as close to the brain as possible ensuring relatively symmetric multi-color illumination.

**Dual Mice Stage**

For the second aim of this thesis we wanted to build a system that is capable of imaging the brain activity of two mice simultaneously while being able to change the distance between the mice. In order to complete this objective, we custom built two identical imaging rigs and placed them facing each other, the rigs are located inside a soundproof custom made box to limit foreign noise confounds in the experiment. The rig on the right was mounted on top of a moveable rail which allows us to change the distance between the rigs. The left rig was mounted on some posts to match the height of the right rig. The stage is built from a milling machine vertical and horizontal translator from Sherline, which gives us the ability to move a platform in the XY direction and
Figure 2.1: Model of the Triple LED Illumination Setup.

Consists of three powerful LEDs with different peaks driven with custom made constant current drivers. Each LED is passed through a bandpass filter to make its emission peak tighter. The three emitted lights are collimated into a tighter beam and the three beams are then combined into a single beam via two dichroic mirrors. At the end the light is focused once again into a smaller beam and put through the liquid light guide. The other end of the liquid light guide is then placed close to the head of the mouse.

focus the camera in the Z direction (See figure 2.3b). The platform itself is built from a piece of plexiglass that is attached to the milling machine stage. On top of the plexiglass platform we have attached the mouse head-fixing mechanism, and the triple LED setup. The mouse head-fixing mechanism consists of three main parts (See figure 2.3a): 1) The aluminum head-fixing posts that clamp the mouses’ head-bar. 2) The plexiglass tube where the mouse rests inside. 3) A 3D printed mount that holds the plexiglass tube and has mounting holes for the bottom plate. The last component of the dual mice imaging rigs is the moveable rail where the right rig sits on (See figure 2.2), the rail is moved by rotating a small setscrew in the direction of movement. In order to move the rail without interfering with the experiment, we use a flexible shaft that we actuate from
outside the soundproof box. The next section discusses the cameras which we attach to the vertical translators.

**Cameras and Data Acquisition**

The dual mouse imaging apparatus operates with two cameras that image the brain activity of the mice, a third infra-red camera images the behaviour of the mice interacting (See figure 2.4). The three cameras are variants of the Raspberry Pi Camera that use the OV5647 sensor by Omnivision, briefly, this sensor is a color complementary metal-oxide-semiconductor (CMOS) chip with a rolling shutter capable of capturing RAW RGB video at 8-bits of resolution per channel (See table 2.1, for camera types). The difference between the imaging camera and behaviour camera is the type of lens they use, the behaviour camera uses a 3.6mm wide-field lens, and the imaging camera uses a 6mm lens with a narrower field of view. In order to block the excitation long blue LED we must add a bandpass filter that blocks that particular blue, but must let through the short blue and the red LEDs and the emission band for GCaMP6s. For that we used the Chroma filter 69013m which is a triple bandpass filter that lets through only the two reflectance bands (short blue and red, details in section 2.2) and the GCaMP6s epifluorescence signal through and into each of the three camera channels. The two imaging cameras are mounted on a 3D printed mount (See figure 2.3c), that attaches to the vertical translator and thus moving it up and down focuses the camera. The focus point of both cameras is then calibrated such that the field of view of the sensor is 8x8mm. We record imaging data at a resolution of 256x256 pixels, and this equates to a pixel size of 31.25µm.

The data acquisition was carried out by three separate Raspberry Pi single board computer (SBC)s Model 3B. The Raspberry Pi is a Linux computer with a 4 core processor, 1GB of random access memory (RAM), 4 USB 2.0 ports, a 100mbit Ethernet port, 40x general-purpose input/output (GPIO) pins and a camera serial interface (CSI) port (See figure 2.5). The Raspberry Pi camera attaches to the CSI port of the Raspberry Pi and the computer handles all the aspects of the acquisition, triggering, data storage, etc. The three Raspberry Pis are used in a master-slave configuration, wherein one of the Raspberry Pis is used as a master and triggers the start of image acquisition.
**Figure 2.2: Model of the Dual Mice Imaging Setup.**

(Top) Digital render of the imaging setup at a distance of 160mm. The mouse on the left imaging setup is referred to as the 'left mouse' and the mouse on the right setup is referred to as the 'right mouse'. It is possible to change the distance between the snouts of the mice from 160mm to 12mm, which allows the mice to engage in a social touch-like behaviour. (Bottom) Digital render of the imaging setup at a distance of 12mm. The right mouse’s setup is mounted on top of a rail, the rail has a handle that shifts the rig when rotated.
Figure 2.3: Individual Models of the Apparatus.

a) The mechanism to head-fix the mice uses a couple aluminium machined clamps to grab the mouse’s head-bar. This image also shows the tube in which the mouse rests, made from plexiglass and a 3D printed tube holder. b) The Sherline milling machine platform mover. The top knob is used to focus the camera, the left and right knob moves the platform in the XY direction. c) The 3D printed camera mount and the Raspberry Pi camera, which attaches to the vertical column of the milling machine platform.
of the other two Raspberry Pis (the slaves). The images of the three cameras are synchronized externally by simultaneously turning on and off the illumination at the beginning and end of the experiment, respectively. This means that the frames in which we see the illumination turn on and off are taken at the exact same time point for both the start and end points of all three cameras. Assuming the cameras do not drop frames and the acquisition clocks do not drift much, this approach is enough to have the data synchronized across the three cameras. For all the experiments (details in Chapter 3), the imaging cameras acquired 24-bit RAW RGB images at a resolution of 256x256 pixels and at a framerate of 28.815 Hz, and the behaviour camera acquired H.264 encoded video files at a resolution of 320x180 pixels and at a framerate of 90 Hz. The Raspberry Pi Camera has its limitations, the camera will drop frames if the data throughput of the video exceeds the speed at which you can save the video to disk. For this reason we have two main approaches to circumvent this issue: 1) Capturing H.264 encoded video at a lower resolution, gives us the ability to capture 90 Hz video without dropping any frames. 2) Saving the timestamp of each individual frame gives us precise quantification of the number of frames that were dropped, and where in time they were dropped. Then afterwards we can interpolate the dropped frames, which works really well for brain activity frames since the spatial information changes little across the individual frames.

The acquisition software consists of two custom Python scripts, one for the imaging cameras and one for the behaviour camera. We utilize the open source picamera library (https://picamera.readthedocs.io/) to control the Raspberry Pi Cameras, we also use the RPi.GPIO library to trigger the two slave Raspberry Pis. We added two main modifications to the picamera library. The first modification was to allow us to timestamp the acquisition time of each frame, and this is used to quantify the timepoints and number of frames dropped, this information is extremely valuable, without it the videos of the three cameras would not be properly synced. The second modification to the picamera library changes the mode of the camera to be linear by disabling the gamma encoding of the CMOS chip, meaning that linearly changing the amount of light to the sensor corresponds to a linear change in the obtained gray values from the sensors. Additionally, the software ensures that the images taken between different timepoints are constant in all aspects:
Figure 2.4: Example Video Frame of Behaviour Camera
The scene of this camera is illuminated by infra-red LEDs, and we capture at a framerate of 90 FPS with a resolution of 320x180 pixels, and the video is immediately encoded using the H.264 codec in order to avoid dropping frames.

<table>
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<th>Part</th>
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<th>Catalog#</th>
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<tbody>
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</tr>
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<tr>
<td>Triple BP filter</td>
<td>Chroma</td>
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Table 2.1: The two types of cameras used for imaging and behaviour.
digital gains, analog gains, auto-white balance and exposure time. To see a flowchart of what the master Raspberry Pi does see figure 2.6.
Figure 2.5: A Raspberry Pi Model 3B and its Main Features.

This powerful and inexpensive SBC is capable of interfacing with many hardware level peripherals making it a very versatile device. Additionally, it is able to interface with a camera, which in our case captures either the behaviour of the mice or their brain activity.
Figure 2.6: Flowchart for the Master Raspberry Pi for Data Acquisition
Chapter 3

Mesoscopic Dual Imaging Results

Methods

Animals

All procedures were approved by the University of British Columbia Animal Care Committee and conformed to the Canadian Council on Animal Care and Use guidelines and reported according to the ARRIVE guidelines. Transgenic GCaMP6 mice were obtained from the Jackson Laboratory, we specifically used the line C57BL/6J-Tg(Thy1-GCaMP6s)GP4.3Dkim/J. A small colony of this line was started at our laboratory. This mouse line expresses GCaMP6s in layers 2/3 of the cortex, and the Thy1 promoter leads to expression predominantly in excitatory neurons [Dana et al., 2014]. From our small colony we used two cages; one cage of four litter-mates and another cage of two litter-mates; however, only three mice in each cage were useable in the cage of four because one of the chronic windows was of poor quality for imaging.

Chronic Window Surgery

Chronic windows were implanted on mice that were at least 8 weeks old, as previously described in [Silasi et al., 2016] and in chapter 1. Briefly, we remove the fur and skin from the dorsal area of the mouse's head, exposing the skull. The area exposed includes the entire two dorsal brain
**Figure 3.1: Overview of Dual Mice Mesoscopic Imaging.**

a) We image the calcium dynamics of two 4.3 Thy1-GCaMP6s mice simultaneously. We vary the distance between their snouts from 160mm down to 12mm. At a distance of 12mm the mice engage in a behaviour that resembles social touch in rats [Grant and Mackintosh, 1963, Lenschow and Brecht, 2015]. We capture raw green GCaMP epifluorescence that reflect calcium levels in the brain, and red and blue reflectance signals that can be used to measure hemodynamics, although we only make use of the blue reflectance. b) We employ mesoscale GCaMP imaging of the dorsal surface of the cortex. At this scale we have access to several cortical areas, the map shown has areas extracted from the Allen Institute Brain Explorer. On the right is an image of what the raw green fluorescence image looks like.

hemispheres and the temporalis muscle at the back of the cranium. We then glue the surrounding skin to the skull using metabond (Parkell, Edgewood, NY, USA; Product: C&B Metabond) glue. A titanium bar, which is used during head fixing, is attached to the posterior part of the skull with the top edge of the bar lying on top of lambda using super glue, then reinforced with clear dental cement. The skull is cleaned with phosphate buffered saline solution and a small drop of very diluted dental cement is added. We overlaid a cut coverslip on top of the skull, gluing it with the dental cement, and then we reinforced the edges of the window with a thicker mix of the dental cement (See figure 1.1). The mice are then allowed to recover for at least seven days prior to any imaging.
Social Touch Experiments

We wanted mice to engage in a social touch like interaction, a behaviour that will be similar to the one previous rats experiments have shown [Bobrov et al., 2014, Ebbesen et al., 2017, Lenschow and Brecht, 2015]; however, we wanted to be able to image the entire dorsal surface of the cortices of both of the interacting mice. To achieve this goal we built two imaging rigs, that were placed facing each other, and henceforth we refer to the mouse on the left rig as the left mouse and the mouse on the right rig as the right mouse. The right mouse’s rig was placed on top of a rail so that we could vary the distance between the snouts of the mice while continuing image acquisition in both animals (See figure 2.2). The distance between the snouts of the mice was changed between a maximum of 160mm (no social touch), down to a minimum of 12mm (possible social touch). Thus, by bringing the mice down to 12mm between each other (while imaging), the mice would engage in a social touch like behaviour, in a similar fashion as the paper by Lenschow and Brecht from 2015 using rats [Lenschow and Brecht, 2015]. We decided to have two have to kinds of experiments: 1) Together experiments and 2) Separate experiments.

Together Experiments

In this type of experiment, we imaged the mice for five minutes and 50 seconds. The mice started at distance of 160mm, and they stayed this way for one minute and 30 seconds. At the 1:30 minute mark we began decreasing the distance between the mice at a rate of 9mm/s, by the 1:45 minute mark the distance between the mice was 12mm. The mice were then kept at this distance for 2.25 minutes and at the 4:00 minute mark the distance between the mice was increased at a rate of 9mm/s. By the 4:15 minute mark the snouts of the mice was back at the initial 160mm. The distance remained at 160mm until the end of the experiment at the 5:50 minute mark.

Separate Experiments

In this type of experiment, we imaged the mice for five minutes and 50 seconds. The difference between the together experiments and the separate experiments is that in the separate experiments the distance was never changed. So the mice were imaged at a constant distance of 160mm for the
entire duration of the experiment.

**Sequence of Experiments**

Each pair of mice were subjected to both types of experiments in the same session. For example, a particular pair of mice would be head-fixed and we would begin with the separate experiment first and then after that experiment is over we immediately start the together experiment. The order of separate vs together experiments would be chosen at random to avoid having some order dependent effect. If a mouse was to be head-fixed again for another pair we would give that mouse a half an hour break before head-fixing the mouse again, this was done to avoid any effect of being head-fixed for long periods of time.

**Image Acquisition**

**Mesoscale GCaMP Imaging**

RAW RGB 24-bit 256x256 resolution frames were captured using the Raspberry Pi Camera, which uses the OV5647 CMOS chip. We recorded with a sampling frequency of 28.815Hz for both the left mouse and the right mouse. The images were captured using custom-written software on the Raspberry Pi single board computer. An additional Raspberry Pi Camera but the NOIR version (infra-red filter not present) was placed beside the mice to record their behaviour (see next paragraph for details). The three cameras were arranged in a master-slave mode, wherein one camera (master) was used to start the acquisition of the other two cameras (slaves). To ensure synchronization of the acquisition, the excitation lights were turned on and off simultaneously, allowing the frames to be synced to the sudden change in illumination. The lens of the camera has a focal length of 3.6mm and was focused at a height that gave a field of view of 8mm x 8mm leading to a pixel size of 31.25µm. The excitation lights consisted of three LED sources where two of them are able to give us information about the hemodynamic changes during the experiment, and the other light source excites GCaMP and we collect its green fluorescence.
**Behaviour Imaging**

H.264 encoded video was captured using the same OV5647 CMOS chip but, with an infra-red version of the camera (for details see Chapter 2). The video was captured at a framerate of 90Hz with a resolution of 320x180 pixels. The scene of the video is a side-view of the left mouse and its interaction zone (See figure 2.4). The entire setup was illuminated with infra-red illumination using two 850nm LEDs, which allows us to record the behaviour of the mice without affecting the GCaMP imaging.

**Image Processing**

**Mesoscale GCaMP Imaging**

The two RAW RGB 24-bit videos corresponding to the calcium activity of the left mouse and the right mouse were imported using the numpy python library on a Jupyter Notebook [Kluyver et al., 2016]. We extract the green and blue channel, which contain the green GCaMP6s fluorescence and the blood volume reflectance signals respectively. The timestamps for each frame of the two video files were also loaded, this gives us the exact moment in time that each frame was captured. We quantify the exact number and location of dropped frames by tagging each frame with a timestamp, and we take advantage of the fact that the spatial information of the imaging videos changes little across time, which means if a particular pixel changes across time it is only due to a change in epifluorescence, so that means we are able to interpolate any frames that are dropped. In general, we do not drop frames, and when we do it is less than 10 frames in a recording of 10373 frames, which means that it is possible to interpolate the signal and reconstruct the lost data. Due to the fact that the GCaMP6s signal changes are quite slow [Chen et al., 2013b]. We then add the interpolated frames to the respective points in time and at the end we have two imaging videos with the same number of frames. We must then extract only the frames where the illumination was turned on. We do this by calculating the gradient of the mean signal across time, the only points where the gradient will be large is when the illumination is turned on or off, and so we can easily remove the dark frames. We then calculate the $\Delta F/F_0$, which is computed by calculating the mean image.
across time (baseline), subtracting it from each individual frame and then dividing this new frame by the baseline. The last processing step is passing the $\Delta F/F_0$ frames through a temporal filter, we chose a bandpass of with a low frequency cut-off of 0.01Hz and a high frequency cut-off of 12.0Hz as most of the information of GCaMP6s can be captured in this band [Vanni and Murphy, 2014]. Finally we save the two processed videos and continue to the analysis pipeline.

**Mesoscale Reflectance Imaging**

The epifluorescence emitted light and the long blue illumination are both absorbed to a different degree, depending on proportion of oxygenated and deoxygenated blood in that area of the brain [Ma et al., 2016b, Pisauro et al., 2016, Wekselblatt et al., 2016]. This means that the readout epifluorescence signal will contain hemodynamics in addition to the calcium dynamics. We try to estimate the contribution of the hemodynamics by the blue reflectance light. We chose the peak wavelength of 440nm because it is close to a point where the difference in absorbance by oxygenated hemoglobin and deoxygenated hemoglobin is negligible (See figure 3.2). In an attempt to decrease the contribution of the hemodynamics to the epifluorescence signal we used a method that has been previously described in a previous paper from our laboratory [Xiao et al., 2017]. Briefly, we process the blue reflectance signal in the same manner as described in the previous subsection, and at the end of the pipeline we take our $\frac{\Delta F}{F_0}$ GCaMP and divide it by $1 + \frac{\Delta F}{F_0}$ blue. Intuitively this means that if the changes in the blue reflectance signal are zero, the epifluorescence signal should not change. This works well enough due to the fact that the hemodynamic changes are relatively small compared to the SNR of modern GCaMP [Dana et al., 2014, Ma et al., 2016a]. The corrected epifluorescence videos are saved.

**Behaviour Video**

The behaviour video is imported using the OpenCV library for Computer Vision, the videos are then decoded and saved as a RAW 8-bit grayscale video file using the Numpy Python library. Finally, using the same method as above, we remove the dark frames. We capture behaviour video at a resolution of 320x180 pixels at 90Hz and we find that we do not drop any frames. Since the
The molar extinction coefficient is a measure of how much light is absorbed by some substance, in this case hemoglobin or deoxyhemoglobin. The spectrum greatly varies with wavelength, we chose to measure the hemodynamics at a point where the difference between the extinction coefficient of both curves is negligible. Image obtained from [http://www.wikiwand.com/en/Photoacoustic_imaging](http://www.wikiwand.com/en/Photoacoustic_imaging)
calcium imaging and behaviour video have different sampling rates we use the behavioural video frames closest to the GCaMP signal in time, that is, every time the acquisition clocks match in time. At the end the three videos are synchronized and always verify this by computing the total capture time in all three cameras and ensuring that they are within a calcium imaging frame of difference (34.7ms). Then we save the resulting file to disk and continue with the analysis pipeline.

**Gradient Triggered Maps**

Gradient triggered mapping is a method where we find simple patterns of behaviour and compute the calcium activity corresponding to those behaviours. The method works by selecting a region of interest (ROI) in the behaviour video where any luminance changes are due to a gross change in mouse posture, whisking or paw movements. We compute the mean luminance across time for that ROI and then we calculate its gradient also across time (figure 3.3a). This results in a vector where large values correspond to points in time where the behaviour was occurring. One particular issue is that these large values are only there temporarily because a particular behaviour may only be present once every couple of frames even though the mouse is still engaging in that behaviour. To improve this response we smooth the temporal gradient of the mean luminance by a convolving this vector with a gaussian with \( \sigma = 20 \). We compute the mean and standard deviation of this gradient and use to set a threshold with equal to \( \mu + \sigma_{\text{threshold std}} \), where \( \mu \) is the mean of the smoothed temporal gradient, \( \sigma_{\text{threshold std}} \) is how many standard deviations above the mean to let through (figure 3.3c). Finally, using this threshold we extract the points in time where the gradient is larger than the threshold and then find the corresponding calcium imaging frames. Then we compute the mean across space of the selected calcium imaging frames (figure 3.3b). This creates a brain activity map that corresponds to points in time where some gross behaviour was happening. For instance, we can draw an ROI across the whiskers of the mice and so the points in time where the gradient is changing a lot will correspond to points in time where the mice were whisking (figure 3.3a-d).
**Whisker Stimulation**

To verify the results of the whisker triggered gradient triggered map (GTM)s we anaesthetized a mouse and stimulated two the sets of whiskers, the macrovibrissae and the microvibrissae [Deutschênes et al., 2012]. The stimulation protocol consisted of 49 stimuli presented every 10 seconds and each stimuli was a one second 12Hz sine wave oscillation that was delivered with a piezoelectric vibration transducer. To calculate the evoked activity map we took 100 frames from before and 100 frames from after the stimulation for each of the 49 stimuli, we then computed the $\Delta F/F_0$ for each of these extracted epochs and we then averaged them together across time. This results in an average response activity video for each of the stimulation protocols. Finally, we calculated the area of peak response for the two videos and compared them.

**Global Signal Analysis**

We imported the two processed imaging video files into a Jupyter Notebook. Then we calculated the global signals of both videos, which refers to an average signal of all the pixels that are considered brain. The delineation of the brain ROI was done by hand, by creating two polygons that outlined the two hemispheres of the brain. Then to calculate the global signal we take the mean of the brain pixels across time. At the end of this pipeline we obtained two vectors that corresponded to the global signal of the left mouse and the global signal of the right mouse. Next we filtered the two global signals at ten different frequency bands: 0.01-0.1Hz, 0.1-0.5Hz, 0.5-1.0Hz, 1.0-2.0Hz, 2.0-3.0Hz, 3.0-4.0Hz, 4.0-6.0Hz, 6.0-8.0Hz, 8.0-10.0Hz, 10.0-12.0Hz. Then for each of these frequency bands we calculated a dynamic measure of signal correlation by using a sliding window and calculating the correlation coefficient between the two signals for each window.
Results

Gradient Triggered Maps Reveal Mutual Whisking Behaviour

We find that the mice engage in bouts of simultaneous whisking between each other (see figure 3.3d) which resembles the social touch behaviour previously shown in rats [Lenschow and Brecht, 2015]. To verify we calculated GTMs using an ROI that captures the whisking from both mice we obtain a map with activity in a posterior region of the lateral cortex. This area corresponds to the macrovibrissae of the mice, which are the longer whiskers that both mice can touch each other with [Deschênes et al., 2012]. We find that these maps are stable across different sets of experiments (figure 3.4), and they only occur for together experiments and not for separated experiments (figure 3.5a,b).

Whisker Stimulation Reveals Similar Spatial Maps as the GTMs

To verify that the area activated by the mutual whisking does correspond to the macrovibrissae area we stimulated the two sets of whiskers (micro and macrovibrissae, see figure 3.6a and 3.7). We find that the area activated by stimulating the macrovibrissae artificially corresponds to the same area when mice whisk at each other. Similarly, the area that is activated when stimulating the microvibrissae differs and is more anterior to that of the mutual whisking. This results makes sense because in the barrel cortex the more anterior barrel fields corresponds to the microvibrissae.

Global Signals Synchronized at 0.01-0.1Hz Frequency Band

When correlating the global signals of the mice across different frequency bands we find that the global signals become highly correlated at specifically the 0.01-0.1Hz band for the together experiments at the moment the mice get close to one another (See figure 3.8a,b). We find that this correlation is not present for the separated experiments, and this difference is significant (See figure 3.8).
Figure 3.3: Mutual Whisking Elicits Posterior Barrel Cortex Activity.

a) The view of the behaviour camera, the white polygon is the ROI where we will detect points in time where whisking occurs. b) The gradient triggered maps for the left and right mouse. We averaged calcium imaging frames where for points in time where a lot of whisking occurred. The blue and red contours were drawn by hand in the area where we saw the largest changes for the left and right mouse. This area corresponds to the posterior barrel cortex of the mice. c) The mean temporal gradient in the white contour from the whisker ROI. We average calcium imaging frames at timepoints that go above the red threshold line. d) The calcium activity from the blue and red contours taken from (b). It can be seen that the calcium signals have large changes at timepoints where the gradient is also large. Furthermore, notice that the signals in the left mouse and right mouse change simultaneously, this is the mutual whisking behaviour that resembles social touch.
Global Signal Synchronization Not Explained by Hemodynamics

It could be possible that the synchronization between the global signal of the mice is not real calcium activity and is in fact explained by hemodynamic changes. To answer this question we performed the same type of experiments on a Thy1-GFP mice line [Feng et al., 2000]. The fluorescence changes that would be observed in this line would not reflect calcium changes in the neurons but instead would reflect hemodynamic changes [Ma et al., 2016b]. We find that there is no correlation between the mice at all frequency bands for both types of experiments (See figure 3.9). Therefore we can feel confident that the at the very least the global signal synchronization
Figure 3.5: Separate Experiments Elicit a Different Map. a) The GTM from a separate experiment looks different. Although spatially it may have the same area activated, the degree to which it activates is different. Additionally the mouse on the right does not show any response to the ROI because the mouse was unable to interact with the left mouse which is where the ROI was drawn. b) The behaviour camera ROI for the separate experiment, we selected the actual whiskers since it would most easily detect them moving.

we observe in the GCaMP6s mice is real calcium activity.
Figure 3.6: Macrovibrissae Stimulation Reveals Similar Map as Together Experiments.
a) Schematic of the location of whisker stimulation, the macrovibrissae. For stimuli we used a one second 12Hz sine wave delivered by a piezoelectric vibration transducer. b) Collage of average calcium activity for all trials. The curve shows the activity in the white contour of the last panel. The peak activity map on frame +25 is in the same location as the whisking GTM of together experiments.
Figure 3.7: Microvibrissae Stimulation Elicits a Different Map. a) Schematic of the location of whisker stimulation, the microvibrissae. For stimuli we used a one second 12Hz sine wave delivered by a piezoelectric vibration transducer. b) Collage of average calcium activity for all trials. Notice the peak area of activation is located more anterior to macrovibrissae area.
Figure 3.8: Global Signals Synchronized at Specific Frequency Band. a) Example of global signals synchronizing when the mice come together. The plot displays the global signal of the left mouse and the right mouse. Synchronization does not seem to last the entire duration of their togetherness (the green shaded region). b) A matrix that shows the sliding window correlation at different frequency bands. The matrix on the left is for the separate experiments and the right plot shows the together experiments. The main frequency band that shows synchronization is the one at 0.01-0.1Hz. c) The correlation coefficients are significantly different for the separate and together experiments.
Figure 3.9: Thy1-GFP Mice do not Exhibit Global Signal Synchronization. a) A matrix that shows the sliding window correlation at different frequency bands for the Thy1-GFP experiments. The matrix on the left is for the separate experiments and the right plot shows the together experiments. There does not seem to be global signal synchronization in the GFP line. b) Global signal syncing is not significantly different between the together and separate experiments.
Chapter 4

Cost effective raspberry pi-based radio frequency identification tagging of mice suitable for automated in vivo imaging.

Introduction

Animal identification is a critical part of any basic or applied research, but correct animal identification is subject to human error. In research that uses rodents, ear clipping, toe clipping, or tail marks can be used to identify individual animals [Wang, 2005]. Of these methods ear tagging is the least problematic from a welfare standpoint, but the marks can become ambiguous over time leading to errors in identification. Furthermore, in cases where there is pooling of cages, duplicate IDs may exist necessitating additional ear tagging that can be stressful for adult animals.

We propose a cost effective system that utilizes RFID to identify mice. A USB based RFID reader interfaces with the RFID tags that are implanted in the mice. The RFID reader is compatible with any USB enabled computer although the raspberry pi is arguably the most cost effective option. A previous method [Gruda et al., 2010], utilized light activated microtransponders; however a disadvantage of this method is that the animal must be immobilized by scruffing which is not possible as part of a self-directed behavioral assay as we have previously described [Murphy et al.,
There are commercially available animal RFID tags and readers such as the FriendChip and those from Kent Scientific, however these are not cost effective solutions and, due to their proprietary nature, cannot be as easily integrated into custom applications. The system we employ can be deployed on all major operating systems and can be readily incorporated into more complex behavioral assays. While other research-based animal RFID tagging systems have been described [Catarinucci et al., 2014, Howerton et al., 2012], our method is aimed at a cost effective solution employing open source software that will facilitate adoption by the community in applications such as automated imaging or tracking animal weights during tasks where food or water restriction is employed as motivation for a specific behavioral task.

Methods

Hardware Overview

The system uses inert glass-encapsulated tags (12.25mm in length) (See figure 4.1A) that are implanted subcutaneously at the nape of the mouse's neck. The unique tags are identified and logged using a USB RFID reader connected to a 3D printed stand (See figure 4.1B). This system of identification has the advantage that duplicates can be eliminated and human errors are significantly reduced. An inexpensive $35 raspberry pi single board computer (See figure 4.1C) can be used to interface with the reader, enabling data collection using the provided, flexible, Python open-source software.

Tag Implantation

All procedures were approved by the University of British Columbia Animal Care Committee and conformed to the Canadian Council on Animal Care and Use guidelines. A cage of five Thy1-GCaMP6 mice (C57BL/6J-Tg(Thy1-GCaMP6f)GP5.5Dkim/J, from The Jackson Laboratory) were used to demonstrate the procedure.

A sterile field was created by placement of a sterile surgical drape. Mice were briefly anesthetized with 2% isoflurane within an induction chamber. Unconscious mice were placed on the
Figure 4.1: Cost effective radio frequency identification reader using a Raspberry Pi computer.
(a) Glass RFID tag that is implanted under the skin over lying the neck. The tag stores a unique number that represents the mouse ID. (b) The USB RFID reader is mounted on a 3D printed platform to hold the reader above the mouses body. (c) The Raspberry Pi computer interfaces with the RFID reader, while connected to a computer monitor and keyboard for manual aspects of data collection such as inputting weights. (d) Command line Python interface that is used to collect the data. Shown on screen are the mouse IDs and the current weight for that mouse.
The surgical drape and anesthesia was maintained with 1.5% isoflurane (See figure 4.2B). A sterile needle and injector (12mm Alibaba Syringe) (See figure 4.2A; see Table 4.1 for all parts) was then loaded with a sterilized RFID tag (soaked in 70% ethanol solution). Note we substituted the tag supplied with the injector for one available from Sparkfun (125kHz glass RFID tag SEN-09416). The thoracic part of the torso was disinfected with betadine and the fur displaced using a pair of forceps to reveal a small patch of skin (See figure 4.2C) that served as the target for the injection needle. An incision was made on the revealed skin using the injection needle and care was taken to ensure penetration of the dermal layers and not the underlying muscle. The needle was pushed anteriorly and parallel to the spinal cord until reaching the nape of the neck (See figure 4.2D). We chose the nape of the neck as mice will be less able to scratch the site of the incision leading to a lower probability of tag-loss and infection. The tag was pushed out using the injector plunger and the needle retracted with the tag remaining in place. The mouse was given buprenorphine (2mg/ml) and was allowed to recover from the injection within their home cage for at least three days before any behavioural training. Alternatively, the mouse can be given an oral tablet of carprofen at 5mg/kg [Kendall et al., 2014] and allowed to recover from the injection within their home cage for at least three days before any behavioural training.

**Electronics**

The RFID tag glass cylinders with a diameter of 1.93mm and a length of 12.25mm were obtained from Sparkfun (SEN-09416). The tags operate at a frequency of 125kHz and encode a 32-bit non-reprogrammable unique ID. The RFID tags were read using a USB based RFID reader (model ID-20LA SparkFun). The SEN-09416 tag was chosen since it is compatible with the ID innovations.

<table>
<thead>
<tr>
<th>Part</th>
<th>Supplier</th>
<th>Catalog#</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFID Glass Capsule (125 KHz)</td>
<td>Sparkfun</td>
<td>SEN-09416</td>
</tr>
<tr>
<td>RFID Reader ID-20LA (125 KHz)</td>
<td>Sparkfun</td>
<td>SEN-11828</td>
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<tr>
<td>Sparkfun RFID USB Reader</td>
<td>Sparkfun</td>
<td>SEN-09963</td>
</tr>
<tr>
<td>RFID Syringe 12mm microchip</td>
<td>Alibaba</td>
<td>1620554840</td>
</tr>
</tbody>
</table>

**Table 4.1:** Parts list for deploying RFID system.
Figure 4.2: Surgical procedure for tag implantation.
(a) The injector used to implant the RFID tag. The needle can be sterilized to inject multiple tags with a single injector. (b) Surgical setup image. Anesthesia was induced in a separate chamber and maintained with 1.5% isoflurane in air using an anesthesia mask (Kopf 923B). The mouse is shown in a stereotaxic apparatus in anticipation of also being used for a cranial window surgery [Silasi et al., 2016], however it is not necessary to employ the stereotaxic frame. In this case the anesthetized mouse is placed on a surgical plate and the injection made while holding the body with the other hand. An experienced operator should take less than 5 min to inject a tag. (c) The incision area was created by displacing fur with a pair of forceps. (d) The RFID tag can be seen slightly protruding at the mouse’s neck as it is being pushed out of the injector.

tag readers also from Sparkfun. We anticipate that other 125kHz glass capsules from sites like Alibaba that match the encoding type and frequency response to that of the SEN-09416 would be suitable. The data was collected and organized using a Raspberry Pi Model 2B computer, using custom software written in Python (see code at http://www.neuroscience.ubc.ca/faculty/murphy_software.html). The software logs the ID of the mouse and combines a precise date stamp and specific event with mouse ID. Although we employ a Raspberry Pi, the system will also run under any PC by installing a version of Python (3.4 or higher) and employing the Sparkfun USB based
Results

Weighing Protocol

Recently several studies utilize water restriction protocols with mice to motivate them to do perform specific behavioral tasks [Guo et al., 2014, Murphy et al., 2016]. Within these studies it is critical to track the weights of the animals to ensure they are in a healthy state, especially if the experiment calls for chronic water restriction. We provide sample software to track of the weights of mice using the implanted RFID tags for identification. The software (see commented Python code at http://www.neuroscience.ubc.ca/faculty/murphy_software.html) is run and a mouse is placed within 25 mm of the tag reader until the program indicates a successful identification (see 3D-drawing of weighing stand at http://www.neuroscience.ubc.ca/faculty/murphy_software.html). The weight of the mouse is measured using a balance and input when queried by the program. The program records the data to text files that depend on the mouse ID, new data is appended to end of file, and the files can be easily backed up automatically using self-served cloud services such as Syncthing (https://syncthing.net/). The software is written in Python and the code is open source and under the MIT license.

The software is written in a modular format. For example, the tag reader class can be imported into a more complex behavioral and/or imaging program. To run the software one has to install Python 3.4+ as well as the pyserial library. One must specify the COM port for Windows or the ”/dev/ttyUSBx” port for Linux systems and then create a TagReader object with the port as an argument to the class constructor. At this point calling the read_tag() function which will return an integer representing the tag ID when a tag is detected. This function can be used in more complex behavioral tasks and can wait for a mouse to be detected before initializing a task parameters specific to that mouses ID.
Discussion

An RFID based animal logging system has several advantages over traditional identification methods. By virtue of being entirely computer based one eliminates most human errors, since the tags implanted in each animal are all unique, one only has to ensure the correct mouse was the one initially selected. The system can reduce animal stress and associated confounds as less researcher contact and handling is required to determine identities. It is even possible to construct systems using RFID-tag coupled gates to completely automate sorting of animals [Santoso et al., 2006, Winter and Schaefers, 2011]. The system we outline is cost effective, a full unit with five tags costs around $85USD. A single RFID reader system can read any number of different tags. One just needs the infrastructure to house many animals and a tag for each animal which cost $5 USD each. It is also possible to link multiple cages together where animals pass through a small chamber allowing their tags to be efficiently read.

The glass-encapsulated tags stay intact beneath the skin of the mouse, without any signs of infection discomfort, or decomposition of the tag. The tags can be recovered and, once sterilized, reused in future experiments when mice have reached experimental endpoints. In an automated imaging experiment [Murphy et al., 2016] the tags remained intact for more than three months with no failures in 16 mice studied.

Although we describe open-source software for weighing, the code can also be used for basis of complex behavioral, imaging, or optogenetic tasks. Recently, we have employed a similar tag reader and tags to trigger brain imaging in individual mice that were automatically head-fixed [Murphy et al., 2016]. Our tagging software and hardware can also serve as the basis of more complex tasks where social interaction can be tracked using RFID tag reader grids, or animals sorted into task-specific compartments within their homecage [Winter and Schaefers, 2011]. Assessment of social interactions in mice is an exciting frontier for neuroscience and has been employed using analysis of depth sensor data using the Kinect Sensor from Microsoft Corporation and the Senz3D depth and gesture sensor from Creative Technology Ltd. In this study individual mice we defined using contrasting coat colors [Hong et al., 2015]. RFID tag reader grids within cages facilitate mon-
itoring social interactions without the ambiguity associated with tracking multiple mice based on video analysis of coat color tags such as paint or bleach, or other strategies which could become obscured with more than two subjects. The RFID tag can also be used to dispense water as in our automatically headfixed imaging task, or extended to dispense animal specific quantities of drugs without the stress of repeated handling [Santoso et al., 2006]. Currently, the Sparkfun reader will read tags within $30.2 \pm 2.4mm$ (n=10) of the sensor, these distances can be modulated by use of shields to decrease efficacy, or a stronger external antenna to read from greater distances. As mice can be moving quickly as they pass the tag reader we estimated the minimal velocity the tag can move to still be read using the standard antenna to be 0.47 m/s. Given this speed in less than the peak running velocity of C57bl6 mice [Billat et al., 2005] one can take steps such as adding obstacles, barriers, or a dead-end chamber to increase read fidelity [Murphy et al., 2016]. Another method to enhance signal is described by ID innovations datasheet (https://cdn.sparkfun.com/datasheets/Sensors/ID/ID-2LA,%20ID-12LA,%20ID-20LA2013-4-10.pdf). The datasheet describes that depending on the power supply used one can add a low pass filter to ensure the output voltage ripple does not affect the AC frequency across the antenna. The data sheet also describes employing a larger, home-made antenna which will also increase the distance read. However, we point that longer read distances are not always advantageous as this can increase cross talk between animals.

One potential issue with multiple tag readers when employed in a grid is interference between the antennas. However, a simple solution implemented in commercial systems is to pulse antennas so that reading is done rapidly but sequentially (Phenosys Social Video system).

**Conclusions**

We anticipate that RFID tag readers and surgical procedures will be the mainstay of any automated behavioral neuroscience experiment and can be used to facilitate the identification of individual animals for imaging assessments or any task needing repeated identification.
Chapter 5

Discussion

The first aim of this thesis was to develop a robust and scalable radio frequency based identification for rodents wherein the mice were implanted with glass RFID tags and the mice were able to be scanned and identified wirelessly. The second aim research aim involves the construction of an apparatus that is capable of imaging two mice at the mesoscopic scale simultaneously, while being able to modulate the inter-mouse distance. We gave an overview of the hardware and software: illumination, data acquisition and stage controller. Finally, the third research aim was to perform sets of experiments where we explore the differences in calcium activity between experiments where the mice were in either of two states: 1) separated 2) together. We will now assess the strengths and weaknesses of the RFID system and the dual mice mesoscopic imaging system. Then we conclude with a discussion of further directions for both of these systems and potential new experiments to further investigate social interactions in mice.

Strengths of the RFID System

One of the main strengths of the RFID system is the ease of use. The glass RFID tags are implanted into the mouse after brief isoflurane anesthesia, the necessary incision is minimal (∼2 mm) and sutures are required to seal the wound. The recovery of the mouse occurs within 1 hour, the wound closes in a few days and after that the mice are ready to be used in a task. The tags are able to be
scanned from a distance of $30.2 \pm 2.4$ mm and we estimated that the tag can be successfully read when the tag is moving at a velocity of at most $0.47$ m/s [Bolaños et al., 2017], which for most purposes is sufficient unless the mice are running at their maximum speed of $0.6$ m/s [Billat et al., 2005]. The software that we developed was well commented and due to its object oriented design it should be easy to incorporate in automated mouse behavioural tasks as we have done in our laboratory before [Murphy et al., 2016; Silasi et al., 2017; Woodard et al., 2017]. Furthermore, the system is robust for chronic experiments [Murphy et al., 2016; Silasi et al., 2017; Woodard et al., 2017] as the tag stays intact for many months. The system is also cost-effective, costing around $85USD for a set of 5 glass tags and the RFID reader each additional tag costing $5USD.

**Weaknesses of the RFID System**

Although the surgery is relatively non-invasive, creating an incision will always come with risks. Although we have not observed any serious complications, there were instances were the mice removed the tag before the incision closed. To improve the outcome we recommend making sure the RFID tag is as far away as possible of the incision and/or add a suture to the incision. Additionally, we recommend that the mice are checked every day until the incision has been totally closed. Another weakness is the difficulty to read multiple mice at the same time as the tags interfere with each other. Work at our laboratory has been done to try to quantify the activity of a group of mice using an array of RFID readers, although the results are not yet satisfactory [Noorshams, 2017]. There is also the fact that the readers interfere with each other when attempting to simultaneously probe for the tags. Nevertheless, we believe that for most research the ability to identify multiple mice simultaneously is not necessary. Other problems arise because the efficiency of reading can vary between tag batches and reading geometry.
Strengths of the Dual Mice Mesoscale Imaging System

Strengths of the Method

The ability to image large expanses of the cortex while two mice come close together is novel. Previous studies have only been able to look at interacting rats while acquiring electrophysiological data from cortical areas such as the barrel cortex [Bobrov et al., 2014; Ebbesen et al., 2017; Lenschow and Brecht, 2015]. Electrophysiological recordings, although they have very fast temporal resolution, the spatial resolution is quite limited to the area around the electrode [Scanziani and Häusser, 2009]. For that reason, being able to image the entire dorsal surface of the cortex allows us to get an idea of what the local and global calcium dynamics are for a social touch-like behaviour in mice. Furthermore, the results of this type of imaging can be used to further guide more in depth electrophysiological experiments.

Modulating the distance between the mice is another strength of this system. A previous study had to manually pick up a rat and bring it close to another rat, which may introduce some confounding factors such as activity caused by the researcher being present in the interaction [Lenschow and Brecht, 2015]. Thus, by placing the entire imaging setup inside a noise reduction box and being able to change the inter-mouse distance while staying outside of the box, we are able diminish additional external confounding factors and standardizing the encounters.

Another strength of this dual imaging system is the small form factor of the imaging cameras. Previously we have employed a different imaging camera for our mesoscopic experiments [Harrison et al., 2009]; however, this camera would not have been suitable for dual mouse imaging because of its large form factor, the camera body itself has a size of 94x94x45mm putting two of these next to each other owuld now allow the mice to come close enough. If we wanted to use that camera, we would have had to develop complicated optical pathways so that the two cameras can accommodate two mice in close proximity.

Finally the main strength of this system is the versatility of the hardware. It should be possible without too much work to add additional behaviour cameras so that one can image in detail a
different view of the mice interacting. Furthermore, due to the small physical footprint of the system it should be possible to add recording electrodes for electrophysiological data or small optical fibers to do optogenectic stimulation without too much issue.

**Strengths of the Experiments**

We conducted two types of paired mouse interaction experiments, one where two mice are imaged at the same time while they are transiently brought close enough to engage in a social touch-like behaviour and the other where the mice stay far away from each other for the entire duration of the imaging session. These experiments are relatively straight forward, the mice are brought to the lab and within 5 to 10 minutes one can have the mice head-fixed and ready to be imaged. Another strength of these experiments is how reproducible the phenomenon is, we observed mutual whisking in every experiment where the mice were brought together and we observed global brain activity synchrony to some extent in 5/6 together experiments. The most important strength of these experiments is probably having the ability to control the distance of the mice, since we do not observe synchrony to the same degree unless the mice were at a close enough distance to engage in social touch-like behaviour.

**Weaknesses of the Dual Mice Mesoscale Imaging System**

**Weaknesses of the Method**

We are limited by the bit depth of the Raspberry Pi Camera, as it is a sensor that sends data in 24-bit RGB format, which means that each color channel sends pixels with a resolution of 8 bits (one byte). Thus, we have a total of 256 possible gray values for pixel intensity and assuming a noise ceiling of zero, we can at most resolve a change of $\frac{1}{256} = 0.4\%$, which is not suitable for other faster sensors with a smaller SNR such as VSD, faster variants of GCaMP and iGluSnFR Madisen et al. [2015], McGirr et al. [2017], Mohajerani et al. [2013]. Although there are a couple of ways to effectively increase the bit depth of the camera: by summing across time on a pixel by pixel basis, though this decreases the overall sampling rate of the signal Harrison et al. 2009]. The second
way is by adding the values of four neighbouring 8-bit pixels and converting it to a single 10-bit pixel, which has the negative effect of reducing spatial resolution.

Another weakness of this system is that the inter-distance mouse is changed manually, although it is possible to move the mice at a constant speed, at this point it is not possible to study the effects of varying the speed as it would have too much variance.

**Weaknesses of the Experiments**

The main weakness of the experiments is that with the current set of experiments that were performed it is not possible to understand what the global signal synchronization means. There does not seem to be an overt behaviour that correlates with this synchronization at such low frequency band. Another weakness of the experiments is that we used GCaMP6s a sensor that has a relatively fast rise time (< 100 ms) but slow decay time (≈ 360 ms) [Dana et al., 2014, Madisen et al., 2015]. This means that the calcium dynamics we are observing may not be reflective of the true underlying electrical brain activity, being able to use an optical imaging method that reflects more closely the true brain activity requires faster sensors like VSD and iGluSnFR which measure voltage and glutamate changes respectively [McGirr et al., 2017, Mohajerani et al., 2013]. The last weakness of the experiments stems from the restraining of mice. Our setup necessitates that the mice be head-restrained in order for them to be imaged and positioned properly. In a previous study it has been mentioned that mice initially find head-fixation to be aversive, but with enough training and habituation their stress levels go down [Guo et al., 2014]. Nevertheless, if our mice were to be stressed during the experiments, then the observed dynamics may not be reflective of true natural social touch behaviour.

**Future Directions for Dual Mice Imaging**

The power of mesoscale imaging in the context of social interactions is largely unexplored. We have only scratched the surface of what system is capable of. For instance, we could employ more advanced computational methodology to analyze high-dimensional brain data and tease out the
behaviour that is associated with the global signal synchrony, in a similar fashion as the recent papers from Dr. Anne Churchland and Dr. Matteo Carandini (Musall et al., 2018, Stringer et al., 2018). In these studies they used linear and latent factor models to discover that most of the neuronal activity variance can be explained by mouse movements and their pupil diameter. To further explore activity associated with this behaviour the possibility of combining mesoscopic imaging with electrophysiology exists. In a similar way as a technique previously published by our laboratory (Xiao et al., 2017), in which the mice are imaged while sub-cortical or cortical electrodes are recorded. In fact, guiding electrophysiological recording sites based on our current results is the most straightforward way to further explore social touch in mice.

Previous research has shown that artificial activation of the dopaminergic neurons of the ventral tegmental area that project to the nucleus accumbens causally modulate social behaviour in mice (Gunaydin et al., 2014). Therefore an interesting experiment to do would be to optogenetically stimulate the same population of cells and observe if the global brain signals synchrony still occurs with out social touch paradigm. A potential improvement to the setup itself would be to improve the sensitivity and SNR of the camera sensor, which would allow us to use faster brain activity sensors in an attempt to image activity that more closely resembles true electrical brain activity, since the current fast sensors have a lower SNR. Another change to the setup would be to allow imaging other types of behaviours, for example imaging the sniffing of the ano-genital region which is a behaviour associated with mating (Grant and Mackintosh, 1963), thus exploring whether or not there are any sex differences and not just imaging male-male interactions. Finally, exploring whether any differences exist with this social touch paradigm across different mouse strains would be very useful for understanding social disorders. For example comparing mice that exhibit autistic-like phenotypes such as the Shank3 or ProSAP1/Shank2 mutant mice (Peça et al., 2011, Schmeisser et al., 2012). In particular looking at any differences in the number of times the mice engage in social touch or any differences in the strength of synchronization of the global brain signals.
Concluding Remarks

We have only scratched the surface with the current setup we built and the set of experiments we performed. Although the results we obtained are limited in some ways, we believe it paves the ground for a plethora of new studies and potential modifications to the setup so that new questions can be asked and answered. We acknowledge that the hardware we used and the mice we chose can be considered limitations, nevertheless we still believe the results are of great interest and useful to guide new questions.

We have managed to complete all the aims that were proposed at the beginning of this thesis, and we hope that someone out there will continue to further investigate social interactions of mice in experiments that combine the data obtained in the mesoscopic experiments and guide cellular experiments. Thank you for reading.
Bibliography


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Appendix A

Supporting Materials

Differences in Cage-mates and Non-cage-mates

In a previous version of the experimental setup described in Chapter 2, we did not have the ability to change the distance between the mice. So the distance between the mice was always a constant 5mm. We performed different sets of experiments where we paired mice that were cage-mates and mice that were non-cage-mates to see if there are differences in their connectivity matrices. A connectivity matrix is a method that has been previously used in our laboratory [Lim et al., 2014]. Briefly, it is a method where we compute the correlation of different areas in the brain across time. So in our case we computed the connectivity matrices for each mouse, but also between two mice. Then we compared the results of experiments that had cage-mates pairings and non-cage-mate pairings. We ran a total of 42 cage-mate pairing experiments and total of 27 non-cage-mate pairing experiments.

For this experiment we used the Ai-94 mouse line, which expresses very well across all cortex as well as in the olfactory bulbs [Madisen et al., 2015]. We found that there seems to be a lot of correlated activity between the mice in their olfactory bulbs for both types of experiments. Additionally, we find that there is more correlated activity in the posterior barrel cortex of the non-cage-mates pairings. When we further investigated those cells of the connectivity matrices we observe that they occur when the mice are engaging in the social touch like behaviour. So it
Figure A.1: Connectivity Matrices for Cage-mate Experiments.

a) For each experiment pair we calculated the connectivity matrix between several known cortical areas. We performed a total of 42 cage-mate experiments where the mice were always at a distance of 5mm. Then all the 42 connectivity matrices were averaged and we display the average correlation coefficient and its standard deviation.

could make sense that you see more bouts of social touch when the mice do not know each other and they are investigating the novel mouse. Unfortunately the reason we did not include this data in the main body of the thesis was because the bouts of mutual whisking usually occur when the mice are first put together as we found out after we modified the setup to modulate the inter-mouse distance.
a) For each experiment pair we calculated the connectivity matrix between several known cortical areas. We performed a total of 27 non-cage-mate experiments where the mice were always at a distance of 5mm. Then all the 27 connectivity matrices were averaged and we display the average correlation coefficient and its standard deviation.