A Framework for Understanding Small Nervous Systems

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A Framework for Understanding Small Nervous Systems

A dissertation presented
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A Framework for Understanding Small Nervous Systems

Abstract

How do we find important neurons and understand how their dynamics control behavior? Both of these are challenging and general problems in neuroscience. In this dissertation supervised by Sharad Ramanathan, I developed a framework to efficiently search for essential neurons and verify them individually using precise perturbations and measurements. I applied this framework to the small nervous system of the nematode *C. elegans* and uncovered neurons that control speed. Working with Dr. Ching-Han Shen, Dr. Josselin Milloz, and Tim Hallacy I screened through 41 transgenic lines that covered more than 82% of the nematode nervous system and uncovered a sparse set of three neurons which affect speed when inhibited. To measure their dynamics in freely moving animals, I worked with Dr. Askin Kocabas and Abdullah Yonar to develop a real-time image stabilization microscope that enabled me to stimulate individual neurons and probe their dynamics on the order of an hour which previously was not possible. I also developed a frequency division multiplexing imaging system which can read out pixel information faster than traditional CCD cameras. Using these tools, I showed that the dynamics in the sparse uncovered neurons were correlated with speed and also observed novel slow dynamics on the scale of tens of minutes in several other neurons. In summary, this study provides a framework to identify and study circuits essential for controlling behaviors in small nervous systems.
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challenging times and she has taught me something that is often difficult to do in our world, to listen. And last but not least, my sister is one of the toughest people I know. She has shown resilience and perseverance in the most challenging settings while simultaneously supporting her friends and family. She deserves the very best.
To

my parents Ho-Shang and Mei-Li Lee
Chapter 1. Introduction

1.1. Approach to understanding neurons and circuits

One of the primary goals in neuroscience is to understand how neurons and their circuits control behaviors. Answering this involves, among many others, understanding circuit architecture and the kinds of computations they are capable of. Trying to understand the whole brain at once, however, is a daunting task. It is technically challenging to simultaneously measure and understand the dynamics of all neurons in small nervous systems such as that of the nematode Caenorhabditis elegans, and seemingly impossible for higher organisms such as humans which have roughly 100 billion neurons. Although efforts have been made towards such a “whole-brain” approach\textsuperscript{1–4}, the neuroscience field has traditionally taken a divide-and-conquer approach and focused on only a small subset of the nervous system at a time\textsuperscript{5}. The hope is that the complex problem of understanding the nervous system can be broken into simpler ones and that the circuits and computations underlying at least some behaviors can be better understood by studying a subset of neurons which are essential to function\textsuperscript{6,7}.

While it is possible that all neurons are functionally tightly connected and breaking any one piece would destroy the nervous system’s functions, we are continually finding examples where this is not the case. A spectacular example is that of the American railroad foreman, Phineas Gage, who in 1848 survived having a large iron rod driven through his head, destroying much of his left frontal lobe. Amazingly, Phineas was able to retain much of his brain function and even operated as a stagecoach driver for several years after the injury\textsuperscript{8}. Such stories suggest that not all neurons are necessary to control
any given behavior. Instead, the picture seems to be that behaviors are encoded by subsets of the nervous system. For example, a recent functional screen of chemosensory neurons in *C. elegans* found that neurons represent their environment using hierarchical sparse codes and that few neurons participate in encoding multiple cues. Activity in sparse sets of neurons has also been a hallmark feature of cortical and hippocampal function. The question then is what are the essential neurons and circuits and how does one go about identifying and studying them?

The success of studying essential neurons has been concomitant with the development of tools and techniques to both perturb and measure neuronal activity. Perturbing individual cells which are micron in size and quantifying their dynamics is difficult. In addition to requiring spatial resolution, measuring individual neurons entails probing dynamics whose relevant timescales could range from milliseconds to hours depending on the behavior and organism. We have come a long way since the early voltage clamp measurements on axons in squids. Perturbation and measurement techniques that used to involve invasive surgery can now be done using optogenetics and imaging. These tools have enabled us to study small circuits of the brain in detail and revealed numerous key circuits such as those controlling turning and bout behaviors in zebrafish and that feed-forward circuits control odor discrimination in rodents. They have been particularly successful *C. elegans* which has several characteristics that make it a model organism for understanding small nervous systems.
1.2. *C. elegans* as a model organism for studying small nervous systems

The nematode *C. elegans* is a model organism for studying how neurons control various behaviors and for developing the tools to do so. The roughly 1mm long worm was the first to have its genome sequenced and the developmental fate of every single one of its roughly 1000 somatic cells mapped\textsuperscript{20,21}. The connectome of its small nervous system of 302 neurons was fully mapped more than thirty years ago and is highly conserved across individuals\textsuperscript{22–25}. Although there are likely some complications, this constancy and repeatability justifies the *divide-and-conquer* approach; it is possible to build the functional map of the worm piece by piece and important neurons found in one worm should be the same in any other. The nematode’s transparency also makes it ideal for imaging and manipulating with optics. Despite its relatively small nervous system, *C. elegans* exhibits complex behaviors such as chemotaxis, mating, learning, and social behaviors\textsuperscript{26–28}. While the neuronal activity patterns are different from that of higher organisms, notably because worms do not exhibit action potentials, there are similarities in architecture\textsuperscript{29,30}. For instance, theoretical studies have identified feed-forward motifs and clustering patterns in *C. elegans* which are also found in the mammalian cortex\textsuperscript{31,32}. Studying the nervous system of the nematode, therefore, may help us better understand small nervous systems as well as the human brain.

Evidence has shown that several *C. elegans* behaviors can be controlled by sub-circuits of the nervous system. Single-neuron ablation experiments have shown that a sparse set of key neurons may be essential for a given behavior, but not others. Conversely, some neurons are involved in multiple behaviors. For instance, behaviors such as egg-laying, defecation and feeding are controlled by a small number of
specialized motor neurons$^{29,33,34}$. These circuits have usually been uncovered by combining the results from functional perturbation of individual neurons with the anatomical wiring diagram. The role of various sensory neurons in encoding mechanical, thermal, and chemicals has been functionally verified$^{35–38}$ and even circuits controlling navigation and chemotaxis from the sensory, to the interneurons that integrate the signals, and down to the command interneurons and motor neurons have been proposed$^{39–41}$. Kocabas et al. found that by synchronizing the activity of a single interneuron to head bending using optogenetics, they could make worms evoke chemotactic-like behavior. Motivated by its success, there has been a continued effort to find essential neurons and modules controlling other behaviors.

Discovering essential neurons and measuring from them has traditionally been difficult. Before measuring and perturbing neurons, one has to first know which neurons to look at and it is difficult to rely on solely on the anatomy. Although there are only 302 neurons in the worm, they are highly interconnected with each directly connected to roughly 15% of the brain through synapses or gap junctions and no two neurons are more than three connections apart$^{42}$. There are a large number of possible circuits and pathways one can draw between any two neurons. And even though neurons can be divided into the three functional classes based on morphology: sensory, inter, motor$^{22,43,44}$, we are continually finding neurons that have multiple modalities$^{45,46}$. Furthermore, even if a neuron is not directly responsible for transducing or modulating a signal, it could still play an important role as a signal channel and integration hub. Therefore, the connectome and anatomy does not reveal what the essential neurons are and we have had to rely on functional experiments. Functionally testing sparse sets of neurons, typically one at a
time, in the large space of possible neurons is challenging in small nervous systems such as that of the nematode and nearly impossible in higher organisms. Improving efficiency and throughput would allow us to explore more of the nervous system. Furthermore, even if we manage to identify the essential neurons, measuring their dynamics is also a challenge.

To understand how essential neurons control behavior, it is necessary to measure their dynamics in a freely behaving animal. This is a general challenge in neuroscience and particularly important in organisms such as C. elegans whose behaviors are characterized by locomotion. The tradition approach of probing with electrodes is being replaced by imaging fluorescent voltage indicators. For C. elegans, one needs the spatial resolution to measure from micron scale neurons in an organism that is moving hundreds of times that scale per second. Furthermore, the timescale of the dynamics that needs to be measured depends on the neurons and behaviors in question and could range from seconds to many minutes. For instance, second-scale resolution is needed to correlate activity with reversal events, whereas tens of minutes are needed to see the slower transition between local-global search. These are outstanding challenges but the work described in this dissertation addresses several of them and allowed me to correlate activity to behavior in a freely moving worm on timescales that previously were not possible.

1.3. Overview of framework

We developed a framework to both identify essential neurons and measure their dynamics. Functionally screening the entire brain for essential neurons one at a time is
challenging so many studies have focused on the same previously studied neurons and behaviors. How then does one generally find essential neurons without knowing where to start? Our approach was to utilize principles of compressed sensing\textsuperscript{52,53} to functionally screen more neurons and in fewer measurements than previously done using traditional single-neuron perturbation methods. To verify and measure dynamics in these neurons, we developed a real-time tracking and image stabilization microscope that enabled us to selectively activate or inhibit individual neurons and measure calcium dynamics in freely behaving animals over tens of minutes. This included developing a fluorescence imaging system that can read out pixels faster than traditional CCD sensory by using frequency division multiplexing.

We applied our framework to find essential neurons controlling speed during chemotaxis in \textit{C. elegans}. Chemotaxis and navigation is widely studied and speed should play an important role because it is relates to how the worm, a point detector, translates space into time. Bees, for instance, trade-off speed for foraging accuracy\textsuperscript{54–57}. Worms crawling on an agar pad towards food show speed fluctuations that range from the order of head-swings, \textasciitilde3 seconds, to many minutes (Figure 1.1).
Figure 1.1: *C. elegans* modulates its speed on timescales of seconds to many minutes. Speed of a nematode crawling freely on an agarose pad. Speed is modulated on the order of a head-swing (seconds) as well as over tens of minutes. Animal occasional slows to a standstill for several minutes as seen at minutes 8 and 15.

We asked what neurons are essential for controlling speed and where these dynamics are coming from. Speed is challenging to study because measurements and perturbations must be done in a moving animal. Using this framework, we functionally screened more than 80% of the nematode’s nervous system and found three candidate neurons controlling speed. Using our microscope, I then verified them by measuring neuronal activity and selectively inhibiting them. Although the developed framework is optimized for the study of *C. elegans*, its principles and techniques may be useful for studying other small nervous systems as well.
Chapter 2. Uncovering neurons essential for behavior

Abstract

I developed a behavioral screen that utilizes compressive sensing to find neurons essential for behavior in fewer measurements than would be necessary using traditional methods. I demonstrated it on a simulated small nervous system and then applied it to C. elegans to uncover neurons controlling forward speed.

2.1. Introduction

Studying import subsets of neurons and the patterns of activity in the circuits they form has helped us better understand how information flows and computations are done within the nervous system to control specific behaviors\textsuperscript{58}. The existence of subsets of neurons controlling a specific behavior suggest that neural networks that perform computations for a specific behavior pass through essential neurons, thus making them central players for that behavior. The ability to identify the specific subsets of neurons experimentally helps us understand the architecture and function. One approach in neuroscience to understand the nervous system is to find key subsets of neurons controlling each behavior through experiments that target specific neural subtypes either through genetics or more recently through optogenetics. The hope is that such understanding can eventually be synthesized to give uncover how the nervous system works at a systems level. Finding the key subsets of neurons that control specific behaviors is challenging problem across many organisms with nervous systems whose
sizes ranging from a few hundred neurons in nematodes to several hundred billion in humans.

Although *C. elegans* has only 302 neurons, they are highly interconnected with each neuron being directly connected via chemical synapses or gap junctions to roughly 15% of the brain as shown by electron microscopy and illustrated in Figure 2.1. The complicated wiring anatomy alone neither reveals how computations are performed in brain nor the identities of neurons controlling specific behavior. Neurons can generally be classified as sensory neurons that have sensory dendrites, motor neurons with neuromuscular junctions, or interneurons that connect the two. This classification gives a rough picture of the flow of information but there are several complications. Naively we might think that neurons which integrate signals from upstream neurons are important hubs and are therefore likely essential neurons. However, because of the high degree of connectivity it is difficult to identify the essential neurons based on just the physical wiring diagram alone. Therefore, one has had to rely on time consuming and challenging functional experiments such as single-neuron laser ablations to find essential neurons.

Although single-neuron laser ablations are possible in several organisms, they have been particularly successful in *C. elegans* and have revealed neurons and circuits underlying several behaviors such as touch avoidance and egg-laying. Ablating any neuron is unlikely to result in a phenotype which implies that not all neurons are important; but ablating a specific neuron can be sufficient. This suggests that information controlling each behavior flows through essential neurons and that these neurons may be sparse in number. Single-neuron functional imaging, which has its own set of challenges, has also shown that chemosensory neurons represent the environment
using hierarchical sparse codes and that few neurons participate in encoding multiple cues. Knowing which neurons are essential can help us focus on important subsets of the nervous system, but finding them is challenging even in one of the most widely studied and characterized model organisms.

The challenges of single-neuron perturbation techniques have made it difficult to sample much of the C. elegans nervous system. Identifying essential neurons has traditionally been tedious—perturbing a single neuron at a time, commonly using laser ablation, and measuring the phenotype for a statistically relevant number of animals is difficult. Furthermore, ablations are typically done at the early larval stage after which the C. elegans nervous system continues to develop and synapses can regenerate, possibly resulting in it deviating from the stereotypical wiring and function. Recently, microfluidics and reversible immobilization techniques have been developed to parallelize laser ablation experiments and increase throughput. Alternatively, neurons can be optogenetically inhibited or activated by driving Archaerhodopsin-3 (Arch) or Channelrhodopsin-2 (ChR2) expression under specific promoters, respectively. Several neurons express unique genes which can be used to optogenetically perturb single cells. In cases where a specific neuron does not express a gene uniquely, of which there are many, it is possible to do intersection optogenetics in which overlapping sets of promoters are combined with the use of a recombinase such as Cre. While these single-neuron perturbation experiments may be simple to interpret, in addition to being experimentally challenging they are the least efficient method of finding sparse sets of essential neurons.
The recent elucidation of compressive sensing from the field of information theory has shown that not only can a sparse signal be compressed, but its information can be acquired in fewer measurements. Given a vector $\tilde{x}$ with $N$ unknowns, finding the $N$ coefficients would take on order $N$ measurements if sampled one-by-one. If the solution is sparse, i.e. most of the $N$ unknowns are zero, however, and groups of them are sampled at a time, the solution can be obtained in order $\log(N)$ measurements using compressive sensing. The critical insight, first published by E.J. Candes and T. Tao, for obtaining the sparse solution from an underdetermined linear system of equation is to minimize the L1-norm, $||\tilde{x}||_1^{52,70-72}$. The commonly used Euclidean distance, or straight-line distance, is the L2-norm, $||\tilde{x}||_2$. Compressive sensing has become increasingly popular in numerous fields such as machine learning, communications, image processing, and genetics$^{73-75}$. Motivated by this and the hypothesis that the set of key neurons is sparse, we constructed a behavioral assay that covered much more of the *C. elegans* nervous system than previously attempted using single-neuron ablation and optogenetics. An optogenetic neural activation screen for over 1000 GAL4 lines has previously been done to construct a brain-wide neural-behavioral map in *drosophila$^{76}$. Instead of sampling neurons one-by-one, we sampled groups of them at a time and minimized the L1-norm to find the sparse solution. I first demonstrated this method using a simulation and then applied it to the library of known *unc* “uncoordinated” genes. I then implemented it experimentally to find neurons controlling forward speed. We used the extensive library of known promoter expression from the literature to drive Arch expression in a handful of neurons at a time. Although the focus of this screen was to efficiently determine the key neurons controlling
forward speed, it serves as a general framework for understanding other behaviors and in other small nervous systems as well.

2.2. Results

2.2.1. Simulation of multiple-neuron inactivation behavioral screen

To test my approach of screening for sparse neurons controlling behaviors, I simulated a small behavioral circuit of roughly 100 neurons with only a handful of essential neurons and asked how well I could recover the identities of the neurons and their strengths by randomly inactivating several neurons at a time and minimizing the L1-norm. Five of these neurons were given non-zero strengths contributing to behavior phenotype when inactivated, while all other neurons were zero (Figure 2.1). As an example, I based the connectivity off the anatomical wiring of *C. elegans*. The simulated network shown in Figure 2.1 thus resembles the nematode’s nervous system with the simplification that all left and right as well as dorsal ventral pairs with the same neuron prefix were grouped as one neuron. For example, SMDDL, SMDDR, SMDVL, SMDVR, were combined into neuron SMD. I then generated data by perturbing random groups of neurons at a time (Figure 2.2), and solved for the sparse solution by minimizing the L1-norm based on the data (Figure 2.3).
Figure 2.1: Simulated network with sparse key neurons resembling *C. elegans* nervous system. Each numbered point denotes one of the 118 neurons in the simulated network. A simulated sparse circuit of 5 key neuron (14, 41, 76, 84, 96) controlling the behavior in question is circled in red. Each key neuron was given a non-zero strength whereas all others are zero. In this example, I modeled the connectivity from the *C. elegans* connectome. Chemical and gaps junctions are shown by grey lines. Adapted from http://www.wormatlas.org/neuronalwiring.html
Figure 2.2: Simulation of inactivating random groups of neurons and measuring phenotype. As an example, 32 different groups of on average 5 randomly chosen neurons are inactivated. The column “Neurons Inactivated” represents the measurement matrix, $A$, and column “Phenotype” represents the data vector, $\vec{b}$, in the linear system $Ax = \vec{b}$.

<table>
<thead>
<tr>
<th>Group #</th>
<th>Neurons Inactivated</th>
<th>Phenotype?</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10, 43, 31, 95, 42</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>29, 1, 100, 16, 8</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>41, 10, 26, 87, 34, 100, 5</td>
<td>+</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>XXXII</td>
<td>99, 78, 39, 8</td>
<td>0</td>
</tr>
</tbody>
</table>

$A$ $\vec{b}$

Figure 2.3: Sparse signal is perfectly recovered using L1-norm minimization. The L1-norm, $||\vec{x}||_1$, was minimized subject to $Ax = \vec{b}$. Not only were the 5 key neurons (14, 41, 76, 84, 96) recovered, i.e. they were non-zero, but their exact strengths were as well.
The method was consistently able to recover the sparse solution but the number of different groups, or measurements, needed to do so depended on the number of neurons sampled in each group. For comparison, the commonly used L2-norm minimization was poor at recovering the sparse signal (Figure 2.4). Typically, around 30 measurements of 5 randomly chosen neurons at a time were sufficient to produce the correct sparse solution—far less than the total number of neurons. On average, finding sparse neurons by sampling one at a time, as is typically done with single-neuron laser ablation, is the least efficient and requires sampling all of them; whereas sampling 5 or 10 random neurons at a time requires 30% to 50% fewer measurements, respectively (Figure 2.5). Sampling more neurons at a time initially results in more false positives, but convergence is faster.
Figure 2.5: Rate of convergence to correct solution depends on the number of neurons inhibited per measurement. Different colored curves show the number non-zero entries found for different sampling sizes as a function of number of measurements. Each curve shows the mean over $n=5$ simulations. Sampling more neurons per measurement initially produces more false positives, but converges faster because it samples the space in fewer measurements. Sampling one by one, shown by the blue curve, has no false positives but is the least efficient and requires the most measurements.

2.2.2. L1 norm analysis of unc genes reveals motor neurons affect uncoordinated locomotion phenotype

I used this L1-norm framework on existing data from the literature to see which neurons are involved in uncoordinated movement. Many genes in *C. elegans*, when mutated, impact the nematode’s coordination and movement are named *unc* genes, or “uncoordinated” genes. The uncoordinated phenotype has typically been observed qualitatively and could range from “flaccid paralysis” to “thrashing” 77,78. Uncoordinated
locomotion may affect speed so essential neurons found for the first may overlap with the latter. Unsurprisingly, I found that motor neurons and command interneurons are important. It is also expected that inhibiting muscle will affect coordination. Each *unc* gene is expressed in 9 neurons on average. I hypothesized that only a sparse subset of the neurons expressing an *unc* gene is responsible for the phenotype. The sparse solution was found by minimizing the L1-norm was composed of 14 of 66 interneurons, of which 4 are command interneuron, 8 of 21 motor, 7 of 31 sensory, and muscle (Figure 2.6).

![Figure 2.6: Sparse solution coefficients and network controlling “uncoordinated” movement.](image)

The sparse solution for neurons controlling “uncoordinated” movement showed a higher representation of motor and command interneurons over inter- and sensory neurons.

2.2.3. *Constructing transgenic lines from known promoters driving Archaerhodopsin-3 expression in groups of neurons*

After demonstrating the benefits of a simultaneous multi-neuron perturbation screen in simulation, I implemented it experimentally by driving Arch expression in
specific neurons using known promoters from the literature. The compounded difficulty of ablating multiple neurons in the same animal, and being able to repeat this for multiple animals, meant that I needed to find an alternative means to perturb neurons. Optogenetic activation or inhibition has the advantage of being reversible and preserving the innate wiring. I chose to use the photoactivatable proton pump Archaerhodopsin-3, or Arch, which when exposed to green light inactivates the neuron it is expressed in, because the blue light used to activate commonly used ChR2 is harmful to *C. elegans* in large dosages. Free online resources such as Wormweb.org have conveniently organized the vast literature on the expression pattern of hundreds of promoters collected by numerous groups over the past few decades. Thanks to the tremendous effort of several colleagues who synthesized and injected the promoter::Arch-tagRFP fusion PCR constructs into hundreds of animals, I screen 41 transgenic lines for a speed phenotype (Table 2.1). The background we used was a *lite-1* mutant which lacks a ultra-violet light receptor and does not avoid light.

The 41 transgenic lines used in the behavioral screen covered 82% of the nematode’s neurons. Due to complications primarily from expression being mosaic, I avoided promoters that were expressed in more than a dozen neurons. Although most of our lines showed the same expression pattern as those from the literature, a handful of them were significantly different, possibly due to slight differences in the promoter construct. We determined the expression pattern for those lines obviously deviating from the literature using high resolution DIC and fluorescence microscopy. As with the simulation, I grouped left, right, ventral, and dorsal pairs as the same neuron. On average, each transgenic line expressed arch in 5 neurons and, as a whole, the screen covered 82%
of all sensory neurons, 62% of all motor neurons, and 89% of all interneurons (Figure 2.7).

![Neuron Coverage](image)

**Figure 2.7: 41 transgenic lines in Archaerhodopsin-3 speed behavioral screen cover 82% of the *C. elegans* nervous system.** All neurons covered by the screen as well as the number of lines expressing them are shown. The screen covers 89% of all interneurons (blue), 81% of all sensory neurons (red), and 62% of all motor neurons.

### 2.2.4. High-throughput behavioral screen

I screen the 41 transgenic arch lines for a speed phenotype using a custom built high-throughput assay. Multiple replicates were done for each line over multiple days. Briefly, a low magnification and large field of view cMOS camera (3-5 frames per second) was used to image between five and fifty animals chemotaxing on a standard 10 cm agar dish. Using fewer animals per experiment enabled to track individuals for longer at the cost of throughput. A 650nm red LED ring light was used to provide dark-field
illumination. Because I wanted to measure behavior, specifically speed dynamics in the context of chemotaxis, a small 10μL droplet of stationary growth phase OP50 bacteria was placed at the center of the dish. At the start of the experiment, groups of animals were placed in small droplets of water 1.5 cm from the bacteria and allowed to freely chemotax towards the food once the droplets dried and released them, typically within a few minutes. On average, >70% of all wild-type animals were able to reach the lawn of bacteria within an hour (data not shown). A powerful 525nm LED light source (~5mW/mm²) was used to activate arch and inactivate neurons. Appropriate color filters were used to minimize exposure to UV-blue light and radiative heating.

Figure 2.8: Archaerhodopsin-3 activated behavioral screen assay.
(a) Schematic diagram of imaging system. 525nm green light from a LED was focused on a 10cm agar dish on which animals expressing arch were placed along with a droplet of bacteria (food). The green light (5mW/mm²) covered >70% of the plate area with less than 15% variation in intensity. Images were acquired at 3-5 frames per second and processed by a PC which also controls the intensity of the green light. Images were
(Continued) processed using custom software from which position and speed, (b) and (c), respectively, were measured. (b) Example of individual *C. elegans* trajectories, shown as different colors, during chemotaxis towards the bacterial lawn (green dot). On average, 70% of all wild-type animals reached the lawn within an hour. (c) Left column: time series of instantaneous speed for individual animals. Right column: histograms of the instantaneous speed.

Behaviors were analyzed from the video recordings using custom software written in MATLAB. Several elements, notably the image segmentation algorithms, were adopted from “The Parallel Worm Tracker” (Miriam Goodman Lab)\(^80\). The centroid position of each animal was tracked throughout the movie and speed was calculated by taking the time derivative. Although not a focus of my study, other behaviors and metrics such as reversals, gradual turning, and acceleration could be easily extracted from the position data (Figure 2.8b). To compare different animals and transgenic lines, I binned the time series speed data into histograms and quantified the difference between them using the Kullbach-Leibler divergence (Figure 2.8c, 2.9). This metric was chosen because I wanted to screen generally for neurons that controlled speed and this could be manifested in higher moments such as variance and skew. For instance, it has been found that dopamine signaling in *C. elegans* controls the coefficient of variation rather than the mean speed\(^81\). The average speed distribution for each line, obtained by pooling the histograms for each replicate equally, was compared to that of the wild-type, \(D_{KL}\langle \text{speed}_{\text{line }i} \rangle || \langle \text{speed}_{\text{wild-type}} \rangle\). A significance threshold was set by measuring inter-animal variability amongst wild-type animals, \(D_{KL}\langle \text{speed}_{\text{wild-type }i} \rangle || \langle \text{speed}_{\text{wild-type}} \rangle\), and taking the 95\(^{th}\) percentile (Figure 2.9b).

To ensure that the light exposure was not inducing any speed phenotypes unrelated to arch inhibition, I cycled the light on (30 sec) and off (30 sec) for many
minutes on wild-type animals and compared their speed distributions. Although we used an ultraviolet light receptor mutant, \textit{lite-1}, which does not avoid visible light, there could be other effects such as photo-damage which could manifest as a phenotype. There was no significant difference in the speed distributions in these control animals when the light was on versus off. The average K-L Divergence ON/OFF (.053 std:.040 n=14, data not shown) was less than the average inter-animal speed variability (Figure 2.9b).

Of the 41 transgenic arch lines in the screen, only 5 (\textit{flp-21}, \textit{npr-4}, \textit{odr-2b}, \textit{sra-11}, \textit{dop-2}) showed statistically significant speed distributions from wild-type as scored by the KL divergence (Figure 2.9). Their mean forward speed were also significantly slower than wild-type. A pan-neuronal arch line, F25B33, was used as a positive control and showed a phenotype. That few lines showed a phenotype is consistent with the hypothesis that the set of essential neurons is sparse. All five lines showing phenotypes expressed in multiple neurons while none of the unique promoters showed a phenotype. Although there has previously never been such an extensive neuronal screen for forward speed phenotypes, there were a few lines that surprisingly did not show a phenotype. My analysis of \textit{unc} mutants showed that motor and command interneurons are important in controlling locomotion which likely affects speed (Figure 2.6). The \textit{unc-25} mutant, as its name implies, has a locomotory behavior in which the animal simultaneously contracts both ventral and dorsal body muscles, resulting in a “shrinking” motion which I expected would have affected forward speed but did not. Previous studies noted that the expression of the transgene is mosaic and phenotypic strength variable which could have made it difficult for my assay to resolve. Similarly, \textit{flp-11} is also expressed in several motor neurons but did not show a phenotype. Interestingly, dopamine and serotonin have
been implicated in controlling speed while crawling on bacteria yet inhibition of serotonin receptor and dopamine synthesizing hydroxylase expressing neurons, mod-1 and cat-2, respectively did not show a phenotype during chemotaxis towards bacteria\textsuperscript{81,83}. Inhibiting dopamine receptor expressing neurons, dop-2 did, however, produce a phenotype. The context of most experiments from the literature was taken on the bacterial lawn or at least near the edge of it and is possibly different from that of our chemotaxis assay.

**Figure 2.9: Speed phenotype for 41 transgenic lines as measured by Kullbach-Leibler divergence.** (a) 5 of the 41 transgenic lines showed a significant speed phenotype as measured by the Kullbach-Leibler divergence between the average speed histogram of the line and wild-type. The blue dashed line, computed in (b), represents the significance threshold and is taken to be the 95\textsuperscript{th} percentile for KL divergence between individual wild-type animals, WT\textsubscript{i}, and the average wild-type distribution, \langle WT\rangle.

After scoring the phenotypes, I used the same L1-norm minimization formalism from my simulation to find the sparse solution and essential neurons. The scoring of
behavior phenotype and neuron expression were both binarized as 1 for phenotype and likely expressed, and 0 for no phenotype and unlikely expressed, respectively. The sparse solution was neurons AVB, RMG, and SIA out of the 97 covered neurons (Figure 2.10a). I chose to binarize the phenotype because with such a large variability in the wild-type control it was unlikely that I had the resolution to treat the phenotype as continuous. Furthermore, because the assumption is that the phenotype producing neurons are sparse, it was unlikely that I would have sampled multiple of them at a time. Therefore the strength of the sparse non-zero coefficients simply scales as the strength of the phenotype. The sparse solution neurons were consistent with literature. AVB has long been known to be a command interneuron driving forward locomotion\textsuperscript{47,59,84}, RMG activation has recently been shown to increase forward speed\textsuperscript{85}, and SIA has neuromuscular junctions\textsuperscript{86}. In contrast, all coefficients in the the L2-norm solution had non-zero strengths and the top 5 percentile, although containing the three neurons from the L1-norm sparse solution, also included the pharyngeal pumping neuron MC which is not expected to control speed and AIZ which has been implicated in reversals but not speed\textsuperscript{87}. 
Figure 2.10: Sparse solution (L1-norm) and L2 coefficients and network controlling forward speed. (a) The L1-norm, $\|\vec{x}\|_1$, was minimized subject to $A\vec{x} = \vec{b}$ where $\vec{x}$ is the expression pattern and $\vec{b}$ is the binarized phenotype. Inset: The synaptic connections between, RMG, AVB, and SIA. RMG synapses onto SIA and AVB via 2 and 3 chemical synapses, respectively. (b) The L2-norm solution.
2.3. Discussion

I developed a framework to identify key neurons controlling behavior. Motivated by the hypothesis that the set of essential neurons is sparse, I sampled the phenotype from perturbing multiple neurons at a time and minimized the L1-norm to find the sparse solution. This order of \( \log(N) \) versus order of \( N \) measurements needed to accurately find the sparse solution is a poignant application of compressive sensing. Although single-neuron ablation experiments have been used for decades and uncovered important circuits underlying several behaviors, their difficulty has limited their search to a relatively small portion of the nervous system. Sampling multiple neurons at a time and using the L1-norm minimization is a more efficient way to find essential neurons. The solution can then be verified using targeted inhibition, such as laser ablation, and functional imaging.

The important neurons that I found capable of controlling speed, RMG, AVB, and SIA, are all consistent with the literature but there were some surprising results which may be due to both technical limitations and my approach. I made the important assumption that any neuron expressing arch at detectable levels was strongly inhibited during the behavioral assay and also that the inhibition was binary—either on or off. The degree to which optogenetically activated rhodopsins can depolarize or hyperpolarize a cell depends on many factors such as their expression levels, the type of neuron, and developmental stage\(^{88,89}\). Prior to behavioral experiments, the animals were screen for brightness as well as characteristic expression pattern. I also chose to use higher light intensity than what is typical to accentuate the perturbation and behavior phenotype. Controls were conducted to ensure that such light did not produce effects unrelated to

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activating arch. Nevertheless, the light levels needed to inhibit may not have been sufficient for all neurons.

Even with the assumption that the essential neurons are sparse, according to my simulations, my 41 promoters should have been enough to get close to the correct solution, but there may be false positives or negatives. For instance, the interneuron IL2 is only expressed in one line, *odr-2b*. This line also expresses SIA which is in another phenotype line, *npr-4*. The L1-norm minimization chooses only SIA instead of SIA and IL2. The possibility of false negatives would decrease by sampling each neuron more frequently (Figure 2.6, 2.7). Sampling neurons more frequently would also increase the number of pairwise and greater groups which would be helpful in understanding epistasis.

The efficiency of this framework over traditional single-neuron ablations could make it possible to measure epistasis and non-linear interactions. Studies in *C. elegans* and zebrafish have shown instances where removing individual neuropeptides had no effect and that they acted collectively where triple mutants did show a phenotype\textsuperscript{90,91}. There may be behaviors that are controlled by essential groups rather than individual neurons. In those cases, all pathways must be shut off to elicit a phenotype. To find neurons with such interactions, it would be necessary to measure all possible pairs, triplets, etc., which is, by construction, not possible with single-neuron perturbation experiments. Furthermore, the search space becomes combinatorially large. For pairwise interactions, each pair can be thought of as an unknown and of which there are $C_2^N$, and goes as $N^2$ for large $N$. Although multi-neuron ablation experiments are possible, it would be prohibitively difficult to search this space by sampling pairs at a time. Using
compressive sensing, however, the search would take of order \( \log(N^2) = 2 \log(N) \) measurements which is much smaller and may be experimentally feasible.

Lastly, optogenetic activation and laser ablation experiments uncover important neurons and circuits capable of controlling behavior, but do not reveal their underlying dynamics. Laser ablation is tonic in nature and while optogenetic activation can be turned on and off, or even varied continuously, it is more efficient to use the behavioral screen to first narrow down candidate neurons and then study their dynamics afterwards. Furthermore, although perturbing these neurons may be sufficient to produce a phenotype, we do not know if the strengths are physiological and we do not know the dynamic range of signals during normal behavior. For instance, although flp-21::arch expressing animals are almost paralyzed under green light, they respond to mechanical touch with reversals and forward speeds qualitatively identical to wild-type suggesting that the optogenetic inhibition was not sufficiently strong to inhibit the neuron under all contexts or that the nematode uses multiple pathways to control speed (data not shown). Perhaps the circuit controlling this avoidance behavior works in parallel to bypass the neurons normally used to control speed during chemotaxis. How the nematode chooses and weighs different pathways could be complicated and context dependent. It is therefore necessary to measure and verify the dynamics in these neurons, using GCaMP functional imaging for instance, while the animal is freely behaving. Functional imaging experiments, however, are technically challenging and low in throughput and therefore should be preceded by such a screen to find a sparse set of candidate neurons as was done in this study.
2.4. Materials and Methods

2.4.1. Strains

Strains were grown and maintained under standard conditions unless indicated otherwise. All experiments were done in lite-1 mutants to minimize the animal’s sensitivity to blue light. All promoters were fused to Archaerhodopsin-3 by fusion-PCR and injected into C. elegans using standard protocols. Animals were fed all-trans retinal (1mM), a cofactor required for rhodopsin activity, for 12+ hours prior to the behavioral assay. All non-transgenic animals as well as transgenic animals not fed all-trans retinal were pooled as wild-type. Day to day variation of the same line showed comparable variability to non-all-trans retinal fed animals of different transgenic lines. Only young adults were used in the behavioral assay.

2.4.2. Promoter expression and neuron identification

The neuronal expression pattern of the promoter:arch constructs was verified using high magnification (100X) fluorescence and DIC microscopy. Only lines showing a phenotype or those with expression patterns that were obviously different from that of the literature under a lower magnification fluorescence microscope were examined. In most instances, a combination of fluorescence-DIC co-localization and neuron process morphology was sufficient to determine the expression pattern. We imaged a minimum of 10 animals for each line and claimed the animal expressed those neurons reliability if they were in at least 75% of them. Although we observed some mosaicism in the expression pattern for several lines, all neurons were expressed in either >75% of the lines or less than <25%.
2.4.3. Behavioral Assay and Image Processing

The green light (5mW/mm²) used to activate arch covered >70% of the plate area with less than 15% variation in intensity as verified with a Thorlabs S120C photodiode. A video camera was used to record the movements of the worms at 3-5 fps. Only data from ~70% of the plate area centered on the bacterial lawn was processed and analyzed. Custom MATLAB software with elements from “The Parallel Worm Tracker” (Miriam Goodman Lab) were used to segment and track the position of individual nematodes across camera frames. To estimate sub-pixel and hence sub-resolution scale displacements, I binned the real space into discrete pixels based on pixel size, i.e. a non-zero displacement was only observed if the animal move more than \( \sqrt{2} \times \) pixel width, the distance between diagonally adjacent pixels. Such analysis is likely more accurate than arbitrary time averaging which is typically used and does not take spatial resolution into account. Under the roughly 1x magnification, each animal was typically around 50-100 pixels in size. In most instances, I was able to track individuals for at least 10 minutes before they either entering the bacterial lawn or colliding with another animal. This time depended on the number of animals used in each trial with ranged from 5 to a few dozen. Speed was calculated by taking the time derivative of centroid position. Forward speed was taken as all speeds at least +/- 3 seconds from a reversal event as identified using characteristic changes in angular velocity.
Acknowledgements

I thank Josselin Milloz and Ching-Han Shen for the designing much of the behavioral screen assay and gathering much of the data. Together with Abdullah Yonar, they made all of the transgenic lines. Jagan Srinivasan and Christopher Chute provided vital assistance to Tim Hallacy in verifying the promoter expression using high magnification DIC and fluorescence images. Sharad Ramanathan came up with the idea to apply the L1-norm minimization to our data and find the sparse solution.

2.5. Supplementary Information

Table 2.1: Promoter Expression Patterns

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Chapter 3.  Measuring and perturbing neurons in freely moving animals

Abstract

In order to verify the key neurons controlling speed found in the arch behavioral screen (Chapter 2), I needed to measure calcium dynamics from individual neurons in freely moving worms with high spatio-temporal resolution. Furthermore, this had to be done over tens of minutes to see slow fluctuations in speed (Figure 2.8c). Existing systems were not capable of doing this so I designed and built a novel real-time tracking and image stabilization microscope that could. This simultaneously enabled me to selectively excite or inhibit single neurons expressing photoactivatable rhodopsins.

3.1. Introduction

Over the past two decades, neurons’ behavioral functions have been identified using a combination of mutant knockouts, laser ablation, and more recently, optogenetics. By driving the expression of Channelrhodopsin-2 (ChR2) and Archaerhodopsin-3 (Arch) under specific promoters and stimulating with blue and green light, it is possible to activate and inhibit, respectively, individual or sets of neurons. Unlike laser ablation, the effects of optogenetics are reversible, non-invasive, and preserve the stereotyped circuit wiring\textsuperscript{68}. Although photoactivating and inhibiting neurons suggests behavioral function, like laser ablation, it does not reveal any of the underlying dynamics. Importantly, it suggests a possible pathway for control but does not prove if it is actually used by the
animal during normal behavior. It is also unclear that tonic inhibition which is mostly commonly used is the appropriate stimulation for studying the nervous system if information is encoded in the dynamics. Furthermore, a combination of high expression levels and intense optical stimulation could drive neurons to non-physiological levels. Electrophysiology, often in the form of voltage clamps, enables the electrical potential to be measured at high temporal resolution but requires dissecting and immobilizing the animal. Imaging intracellular calcium levels with calcium-sensitive fluorescent proteins, most prominently GCaMP, to readout neural activity has therefore become a popular means for measuring dynamics. It is widely used in nematodes, drosophila, zebrafish and mice. Measuring GCaMP activity in freely moving animals, however, has traditionally been difficult.

One challenge of measuring GCaMP in a moving animal is that motion artifacts are prominent at the higher magnifications needed to resolve weak fluorescent signals from individual cells. The vast majority of neurons in C. elegans are densely clustered in the head region around the nerve ring. High magnification objectives, i.e. ≥50x, with large numerical apertures must be used to collect enough light and resolve cells. This consequently limits the field of view to only several hundred microns. C. elegans can easily move at speeds >250 um/sec on an agar pad and traverse the field of view in less than one second, making even minute long experiments impossible without constraining or tracking the worm. Furthermore, the motion in the field of view blurs the image and reduces resolution. The main challenges, some general to fluorescent imaging, of measuring GCaMP in a moving animal can thus be summarized as,
- High magnification, numerical aperture are needed to resolve neurons and signals
- Motion artifacts are pronounced at high magnifications
- GCaMP fluorescence signals are typically weak
- Animals should be freely moving, i.e. unconstrained, to observe natural behaviors
- Excitation light should be minimal to prevent light induced behavioral response and photo-toxicity

Earlier methods for imaging GCaMP in moving worms sought to physically constrain the worms and sacrificed free movement for ease of imaging. Immobilizing worms in glue or by administering sedatives removed motion artifacts all together and enabled high resolution imaging\(^{47,93,94}\). Guo et al. were even able to simultaneously combine ChR2 activation and GCaMP imaging of upstream and downstream neurons, respectively, using spatially structured illumination to decipher functional connections in a stationary worm\(^{47}\). Although these experiments did not allow GCaMP activity to be imaged during behavior they could be linked by combining results from ChR2 and laser ablation experiments in freely moving worms. An extension of this was to image worms in microfluidics devices which allowed the local chemical environment to be precisely controlled and supposedly resulted in less prociceptive sensory response than immobilization\(^{95-97}\). Recently, this has become the platform for doing ‘whole-brain imaging’ in which neuronal activity from 50+ neurons are simultaneously recorded over several minutes\(^{9,98}\). These microfluidics experiments have led to many discoveries such as the odor response profiles of sensory
neurons. It is difficult to believe, however, that this method is significantly less perturbing than immobilizing in glue as the worm’s movement is restricted to just a few microns. Although dynamics in GCaMP activity can be observed, they cannot be directly correlated to behavior, and must be understood in the context of the worm being immobilized. Being able to image in a freely moving worm would solve these issues. Special ‘worm arenas’ have been developed which partially constrain worm motion and image with a lower magnification and larger field of view objective. The forces of swimming in liquid is different from that of crawling on an agar pad and it is not clear if the worm thinks and coordinates neural activity differently in these contexts. Furthermore, in compromising resolution for movement these techniques struggle with signal-to-noise. An alternative to constraining worm movement is to mechanically compensate for it.

Techniques have been developed which mechanically compensate for motion artifacts, i.e. track, but it has not been possible to do high resolution GCaMP imaging in a freely moving worm for more than a few minutes. Here, freely moving, means crawling on the surface of an agar pad—the most commonly studied context and from which most behaviors have been characterized. This has been challenging because the worm’s speed relative to imaging field of view is large and therefore requires both precise and fast feedback. Latency, the total delay between the worm’s movement and system’s response to it, must be minimal. Often mistaken as acquisition time or loop rate, latency includes all the delays in the pipeline from acquisition, data transfer, computation, and mechanical response. Low magnification tracking systems can follow worms for many minutes and even activate individual neurons but lack the resolution and numerical aperture to
measure GCaMP dynamics. Recently, several groups have started to do high magnification and more precise tracking by tracking fluorescently tagged cells rather than the body posture which is constantly distorting during locomotion. These marker neurons are typically much brighter than GCaMP and because they are stable, can be used for tracking. Although these systems have higher magnifications and resolution, they are still not capable of tracking freely moving worms on an agar pad. Not only are they unable to correct for motion artifacts on the scale of the neurons they are trying to image, but they cannot track the worms for the tens of minutes needed to see the slower dynamics that characterize commonly studied behaviors such as local-global range search. Some confine the worms in mineral oil between a coverslip and the agar plate where they crawl and reverse several times slower and more frequently, respectively. Others sacrifice resolution for speed by using a lower magnification such that the worm does not leave the field of view between exposures. Common to all of them is that they use intense light to image GCaMP which is problematic.

One limitation of prior art tracking methods is that, although they keep the worm in the field of view, they all require dangerously high levels of light. Edwards et. al published a report which importantly, given the popularity of optogenetics, showed that *C. elegans* is acutely sensitive to light—particularly to blue and shorter wavelengths which is what excites GCaMP. They showed that just 0.5 mW/mm² of blue (500nm) light evoked an escape behavior. 5.5 mW/mm² of green (545nm) light showed no effect on the worm over the course of an hour but 5 minutes of exposure to that same intensity of blue was lethal. For comparison, current techniques use a minimum of 14 mW/mm² and up to 43 mW/mm² of blue light. In their report, Edwards et. al identified a light receptor...
responsible for the escape response to blue light. Many groups, including ours, use this mutant to eliminate the escape response when using blue light but this does not imply the worm is not being stressed or damaged. Evidence supporting that worms are not stressed or dying in these high light level tracking experiments is lacking. Even if existing systems were technically able to reliably track worms for many minutes, they would probably die within a few minutes due to the high light levels. More worrisome is that GCaMP and the behavioral response measured from these systems might have to be taken in the context of a dying worm. Other calcium indicators, mostly derived from GCaMP, that are excited by longer wavelengths such as YCaMP and RCaMP exist and could be used instead but are dim in comparison.

My goal was to develop a real-time tracking and imaging system that used minimal and safe levels of light. Even with state-of-the-art EMCCD cameras, using more than 10-fold less excitation light while still maintaining good signal-to-noise is difficult unless exposure times are increased. Increased exposure times, however, exaggerate motion artifacts. My approach, therefore, was to track the neurons so quickly and accurately that they not only stayed in our field of view, but looked stationary. This technique is known as ‘image stabilization’ and is widely used in photography, although there the motions from the camera and operator are stabilized, not the object. Not only did this make GCaMP imaging in a moving worm possible, but it enabled me to use spatially structured light to selectively perturb neurons expressing photoactivatable rhodopsins as Guo et al. did in a fixed worm. I was therefore able to inhibit just the essential neurons identified by the Arch behavioral screen in Chapter 2 and verify their function. Targeted
illumination has already been shown, but not in a freely moving worm and with such high spatio-temporal resolution.

3.2. Results

I designed and built a microscope that stabilizes the worms few hundred microns per second artifacts to just a micron and allows GCaMP to be imaged with >30 millisecond long exposures and individual neurons to be photoactivated. I approached the challenges of imaging GCaMP in a freely moving worm by separating tracking and imaging such that they could be optimized independently. Tracking worm movements and imaging GCaMP have inherently different timescales. Tracking should be done quickly with minimal exposure times. Imaging GCaMP on the other hand should be done with long exposure times such that low light levels can be used without sacrificing signal-to-noise. To track with single-cell resolution, I naturally sought to track the cells themselves. I labelled a specific neuron in the head region with mKusabira-Orange. This bright ‘marker’ neuron label is then co-expressed with promoter driven GCaMP. I chose AWCR or AWCL as the marker neuron because its soma which can be used for tracking x,y,z is conveniently located in the head region where most of the sensory and interneurons are and has process that projects anteriorly to the nose tip which can be used for tracking orientation (Figure 3.1).
Figure 3.1: Fluorescence image showing a worm head with co-expressed marker neuron AWCR::mKO and GCaMP expressing neurons. Worm is oriented on its side with its nose pointed to the left. Tracking and stabilizing the movement of the marker neuron simultaneously stabilize the movement of nearby neurons. AWC marker neuron, shown in orange, is located in the head region just anterior to the second bulb. GCaMP or photoactivated rhodopsin expressing neurons were co-expressed and are shown in green.

The microscope I designed and built is optimized for imaging *C. elegans* but addresses several challenges common to imaging other organisms. A worm is placed and allowed to crawl on standard agar plates, as opposed to in special microfluidic chambers, which is not only convenient but also reproduces the conditions used in most behavioral experiments over the last few decades\textsuperscript{59,101–103}. The thickness of the pad and dish limits illumination and imaging to one side and therefore required an epi-fluorescence configuration. Fluorescence from both the marker and GCaMP neurons are collected by the same 50x 0.55NA objective and sent to separate EMCCD cameras downstream for processing. This high magnification and NA objective not only efficiently collects light, but also limits the illumination to the only the region being imaged, thereby reducing total power on the worm. Images acquired by the cameras are read onto separate FPGAs.
for processing and saved onto peripherally connected PCs. A 1x long working distance
and wide-field lens images the worm’s posture from the other side of the dish.

The details of the components and techniques are discussed further in the Materials and
Methods below.
**Figure 3.2: Image Stabilization Microscope.** (a) Diagram of tracking and image stabilization microscope. Key components and light paths are shown. Arrows denote the direction of information flow. The dove prism and position stages both receive commands from the FPGAs and send their positions to them. Timestamps are sent between the FPGAs to synchronize data. The tunable lens assembly used to scan different z positions for GCaMP imaging is shown by the boxed dashed lines. (b) Dual-view image AWC marker neuron simultaneously imaged at two different focal planes onto Andor camera in (a). (c) Z-stack GCaMP images taken by Hamamatsu camera in (a). In both (b) and (c), the worm is oriented with its nose pointed up.
3.2.1. Real-time Tracking and Image Stabilization

Tracking and image stabilization are achieved through a combination of fast acquisition, computation, and mechanical feedback. In order to stabilize worm movement, x, y, z, and $\theta$ coming mostly from head bending, must all be tracked simultaneously. Without tracking x, y, the worm quickly leaves the field of view. Z position must also be tracked because even slight unevenness in the agar pad and head lifts can move the image too out of focus to track x and y (Supplementary Movie S1). Thus, loss of tracking in any one of these dimensions results in losing the worm and all must be done simultaneously.

Briefly, a green 543nm laser (5mW/mm$^2$) is used to excite the mKusabira-Orange tagged marker neuron, AWC. Fluorescence is collected by a 50X 0.55NA objective and passed through a dove prism mounted on a rotation stage. The dove prism utilizes internal reflections to rotate the image. Due to weight and space constraints, I chose to rotate the image rather than the sample itself. The rotated beam is then split into two adjacent images that focus at slightly different z depths on an EMCCD—one in focus, the other slightly out of focus (Figure 3.2b). This step, colloquially referred to as “dual-view”, was accomplished by inserting a slightly focusing lens in one of the otherwise optically equal length paths and is a modern interpretation of a classic technique used by H. Berg to track swimming *E. coli* $^{104}$. The pixels from the camera are then read serially onto the FPGA and processed immediately as they enter.

To facilitate fast and reliable real-time processing, I used Field Programmable Gate Arrays. FPGAs are parallel processing devices that are able to run at much higher and controlled clock speeds than traditional sequential processing CPUs. Once the image is read onto the FPGA, the x, y, z, and $\theta$ positions are calculated in parallel, without any
one process delaying another. The x,y position is calculated from the centroid of the cell body in the focused image. Z position is measured by calculating the difference in intensity between the focused and out-of-focus images. The angular orientation, $\theta$, is calculated from the marker neuron’s process which extends from the cell body to the animal’s nose tip. The displacement from the target position is then passed to x,y,z, $\theta$ stages (x,y: piezo+servo, z: stepper, $\theta$: servo) to mechanically move worm back to the target location. This is all done at 100 frames per second and less than 10 millisecond latency. As a result, we can stabilize the >250um/sec movements of the marker neuron cell body to 1 um—less than its radius. Head bending motion artifacts are also reduced two fold (Figure 3.3). Furthermore, tracking and stabilization can be maintained over an hour and centimeters of travel.

**Figure 3.3:** X,Y position is stabilized to within 1 um and angle bending artifacts are reduced 2-fold. (a) Histogram of centroid position of tracked marker neuron AWC soma from freely moving worm gathered over 5 minutes, as seen on stationary camera. (b) Blue: angular position of dove prism rotation stage, Red: angle orientation of AWC process after dove prism rotation compensation, as seen on stationary camera. “0 degrees” denotes the targeted orientation. The microscope keeps the worm in the same orientation and reduces head bending amplitudes by a factor of two.
**GCaMP Imaging**

Tracking and image stabilization allow GCaMP to be imaged as if the worm were immobilized. I used up to 30 millisecond exposure times without noticeable motion artifacts which enabled me to use blue light levels around only 0.5-1 mW/mm²—several times less than the reported dangerous level and an order of magnitude less than what other methods use. This low light level is likely one of the reasons I have been able to image freely moving worms at high spatio-temporal resolution for an order of magnitude longer than other methods.

Stabilizing the image also enabled me to perform volumetric z scans as can be done on some commercial microscopes equipped with autofocus systems, such as Zeiss Definite Focus and Nikon Perfect Focus, except that those systems track the coverslip surface rather than the sample. These commercial systems can compensate for temperature related z drift but cannot do volumetric scanning in a moving sample. Because the GCaMP imaging and tracking systems share the same objective, the distance between the objective and sample cannot be varied to do z scans. Instead, I utilized the same principle from the z tracking system and placed liquid tunable lens in a 1x relay system between the tube lens and GCaMP imaging camera. The focal length of the tunable lens is voltage controlled and can be varied between 100 to 200mm. In this configuration, the tunable lens can scan through the entire ~50 um width of the worm without any variance in image quality or magnification. Including the 30 ms exposure times, I can scan through the entire worm (15 z-slices) in one second (Figure 3.2c). This bandwidth of 1Hz is sufficient for measuring neuronal activity in *C. elegans* because it has been found using high bandwidth electrophysiological patch-clamping that they show
second-scale graded responses, as opposed to millisecond-scale action potentials in higher organisms such as drosophila and mammals\textsuperscript{30}.

3.2.2. \textit{GFP Controls to characterize motion artifacts and sampling noise}

I used GFP as a control to characterize remaining motion artifacts and noise in the microscope. A specific promoter was to drive GFP expression in a left right pair of interneurons, AIYR/L. Movement artifacts would have the same effect on measuring GFP as it would GCaMP. Similarly, readout and dark noise from the camera would be the same, as would shot noise given that the GFP and GCaMP intensities are comparable. Several techniques therefore utilize ratio-metric imaging in which the GCaMP signal is compared to that of a RFP or DsRed from the same neuron\textsuperscript{3,48,105}. The non-GCaMP fluorescent signal then acts as a control for each worm. One of the dangers of using this technique, however, is that because fluorescent signals are weak, this involves dividing by small numbers which result in large errors. My GFP control shows that not only can I resolve the two cell bodies and process in a freely moving worm over an hour, but the errors in the measurement, including all remaining motion artifacts and camera noise, is less than 18\% as measured by the standard deviation (Figure 3.4b). This 18\% was taken to be my intensity resolution at our typical 15 fps sampling rate and only GCaMP measurements with fluctuations greater than this have a signal-to-noise greater than one and considered to show signal. When smoothed or time averaged over 3 seconds, the GFP intensity standard deviation is less than 10\% indicating that much of the noise we observe is in frequencies faster than 0.3Hz.
Figure 3.4: GCaMP from multiple neurons can be simultaneously imaged with less than 18% error in a freely moving worm over many minutes. The microscope’s ability to resolve GCaMP activity in different neuron soma and processes was characterized using a transgenic line expressing the marker neuron and aiy::gfp. (a) GCaMP images from the z-stack time series of the left soma, right soma, and process of aiy::gfp acquired during the experiment. (b) Time series of intensity from the cell structures in (a) taken at ~1 Hz. The fluctuations represent noise and motion artifacts and the standard deviation, 18%, was used as the error bar for all subsequent GCaMP imaging. (c) Instantaneous speed of the worm with 1 second moving average smoothing. (d) Trajectory of worm.
3.2.3. **Targeted illumination in freely moving worm with spatially patterned light**

To verify that the key neurons discovered from the Arch behavioral screen (Chapter 2) through the L1-norm minimization, I needed to selectively inhibit them to show that this alone produces the observed phenotype. Rather than use laser ablation or intersection genetics, I targeted individual neurons using spatially structured illumination. Using a spatial light modulator such as a DLP to selectively excite specific neurons has been previously done in a moving worm but never at such a high resolution\(^{40}\). The obvious challenge is that one must illuminate a small target that is moving at a several hundred times its size every second. Fortunately, the challenges associated with movement artifacts were solved by the tracking and image stabilization for imaging GCaMP.

Using spatial structured illumination, I was able to selectively excite individual neurons spaced less than 20 microns apart. As a control, I tried to selectively illuminate RMG in a promoter that expressed GCaMP in that and several other neurons in the head region, \(flp-21::GCaMP6s\). Patterns were generated on a PC and sent to the DLP projector via HDMI where the image was then projected onto the worm. Even in an animal moving at more than one hundred microns per second, common for worms moving freely on an agar pad, I was able to excite only RMG which was spaced less than 20 micron away from the M2 (Figure 3.5).
Figure 3.5: Targeted illumination combined with tracking enables single-neurons to perturbed in a freely moving animal. As a control, a flp-21::GCaMP6s transgenic worm was tracked and neuron RMG was specifically illuminated and not M2 and URA. The illumination was altered between dim, uniform illumination and the same illumination plus targeted RMG illumination. The Hamamatsu camera (Figure 3.2a) was used to image the fluorescence. (a) Fluorescence image showing neuron expression of flp-21 promoter. Flp-21::Arch shown here but both GCaMP and Arch had same expression pattern. (b) RMG showed an increased in fluorescence when the structured illumination was on (grey bars) but both M2 and URA were unaffected. (c) Speed of the worm smoothed over 1 second.
3.3. Discussion

Using this tracking and image stabilization microscope we can now for the first time measure GCaMP dynamics in freely moving worms for over an hour. Imaging GCaMP in a freely moving animal has been a goal for many years. Starting with immobilization, it quickly developed into partial immobilization using microfluidics and then tracking. Although several systems can track a worm freely crawling on an agar plate, none have the ability to do image stabilization. This entails stabilizing movements to within microns in a worm that can move at more than 250 μm/sec. Without image stabilization, these systems have had to rely on fast exposures and consequently high light levels which leads to several issues—the primary one being that worms die under intense exposure to blue light.

By separating the challenges of imaging from those of tracking and stabilization, I was able to address them independently with little compromise. At the ~50X magnification needed to resolve single neurons, I needed to simultaneously track x, y, and z movements to within microns. I solved this by focusing on speed and reliability, using state-of-the-art components wherever possible and custom fabricating those that weren’t available. The necessary fast computations, for instance, had to be done on FPGAs rather than traditional PCs. As a result, the tracking and image stabilization was effective enough to allow me to use 30ms exposure times to image GCaMP and reduced the blue light levels to where I do not see any signs of photodamage or stress on the worm even after an hour of imaging.
Compared to existing methods, ours has several advantages. The low blue light levels and ability to measure for tens of minutes make it the only system capable of observing slow behavioral dynamics such as the local-global search transition\textsuperscript{51}. Although I have found that illuminating the entire worm using my typical light levels does not damage the worms, I could use the targeted illumination to reduce the total exposure if necessary. Worms are also imaged on standard 10 cm agar petri dishes which is not only convenient, but replicates the same environment in which most behavioral observations and experiments have been done over the past few decades. Microfluidics, although useful for delivering chemical stimulants, place the worms in a different context and care should be taken when combining results from the two. The extent to which being in a microfluidic device affects decision making and behavior has to be further characterized but it is currently known that worms reverse more in the devices\textsuperscript{3,97}. Our system also allows us to deliver odors on the worm and we can do closed-loop feedback experiments such as head synchronized turning (data not shown)\textsuperscript{40}. The convenience and reliability of the microscope also enables us to increase imaging throughput—we can easily image a dozen individual worms in a work day.

Although optimized to track and image \textit{C. elegans}, our method uses tools and techniques applicable to other fields such as \textit{in vivo} imaging in mouse and zebrafish. The general challenges of fluorescent imaging are the same. Larval zebrafish are transparent, which like \textit{C. elegans} makes them convenient for doing GCaMP imaging. Their movements, especially the bouts, are much faster and therefore imaging can currently only be done in fish that have their heads embedded in agar\textsuperscript{1,15}. Fortunately their tail movements read out their intended swimming behavior but having their heads fixed could
still stress the animal and affect motor feedback. Although our system is unlikely to be able to track and stabilize the bouts, it may be sufficient to follow them at more dormant times. Imaging GCaMP even in anesthetized mice is difficult because the motion caused by breathing is significant. Mice have, therefore, traditionally had to have their heads fixed during imaging\textsuperscript{18}, but recently there has been some use of fluorescent beads as landmarks for tracking\textsuperscript{106}. The use of z-tracking from our system could solve this motion artifact.

Future work includes several technical improvements as well as exploring the use of other marker neurons to image different regions of the worm. The length of the light path from the object to the camera is roughly a meter and shortening it would increase light collection efficiency. This would mean shorter exposures and faster speeds, and/or decreased excitation light. Currently, the path length is limited by our use of off-the-shelf optical components but using custom fabricated ones can reduce it by a factor of at least two while also improving stability and adjustability. We chose to use AWC as our marker neuron because of its location in the head, but because of the worm’s distortion during locomotion that cannot be corrected by x,y,z, and rotation alone, tracking and image stabilization is worse for neurons further away from AWC. This could be solved by using different marker neurons situated close to the neuron(s) we want to image.

3.4. Materials and Methods

3.4.1. Tracking

\textit{Dove prism for image rotation}
I used a dove prism mounted in a rotation stage to rotate the image and compensate for the worm’s head bending. Dove prisms have been used in astronomy and machine vision as ‘beam rotators’\textsuperscript{107}. A challenge of aligning the dove prism is that its rotation axis must be collinear with that of the rotation stage. Small lateral or angular deviations result in image nutation, or ‘rocking’. Fortunately, there are some established alignment procedures\textsuperscript{108–111}. I assembled an alignment apparatus using a combination of off-the-shelf stages but custom holders and shims should ideally be used to reduce weight and bulk. Dove prism (Thorlabs PS992-A).

![Figure 3.6: Illustration of a dove prism.](image)

**Figure 3.6: Illustration of a dove prism.** An image passing through a dove prism undergoes several internal reflections and exits rotated 180 degree. Note that rotating the dove prism an angle $\theta$, rotates the image $2\theta$. Reproduced from osa.magnet.fsu.edu.

**Z tracking from two focal planes**

I tracked $z$ position by comparing the fuzziness between two images of the marker neuron taken at different focal planes. The technique was first utilized by Berg in 1971 to track swimming *E. coli*. The fuzziness of an object, as measured by its intensity, does not give the absolute position as the fuzziness is symmetric above and below the focal plane. Comparing this to the image from a different focal plane, however, allows the exact $z$ position to be calculated as the difference in intensity between the two images is
monotonic near either focal plane. Berg explains, as the object moves toward one focal plane, “its image sharpens on [that focal plane] and fuzzes out more on [the other plane].”

\[X,Y,Z,R\] stages

To get precision and speed, along with range, I stacked fast but small \(x,y\) piezo stages (PI) on long travel servomotor stages (Newport LTA-HS). Micron-scale position was read from the encoded stages onto an FPGA (National Instruments PXIe-7966R) as an analog voltage. A non-encoded linear stepper motorized stage (Thorlabs ZFS13B, Sparkfun EasyDriver) was used for controlling \(z\) position. The dove prism used for rotating the image was placed in a servo controlled rotation stage (Newport URB100C) which was set to track a voltage and corresponding angle using its own internal PID feedback. The servo stages were all driven by a stage controller (Newport XPS Controller) and custom written .tcl scripts. All tracking movement signals were processed and sent by an FPGA. Custom built digital-to-analog and analog-to-digital converters (Harvard University Electronic Instrument Design Lab) were used wherever necessary.

\textit{LabVIEW FPGA for hardware control and real-time processing}

Field Programmable Gate Arrays were used to control both tracking and imaging. Essentially low-level programmable hardware, FPGAs offer several advantages over traditional CPUs for facilitating image acquisition, processing, data transfer, and signal output. Processes on FPGAs can run in parallel as opposed to sequentially on a CPU. This means that each process loop runs exactly at its specified rate no matter the loading
on itself or other loops. FPGAs are therefore widely used in applications that require precise timing. They are especially useful in image processing because as pixel data is transferred one after another from the camera, it can be simultaneously processed by separate processes to apply various filters, and calculate $x, y, z$, and $\theta$ positions for example. I used a National Instruments 1483 Camera Link Adapter Module to interface the camera (Andor iXon Ultra 897) to the FPGA. The different stages have different sampling rates and because the FPGA can communicate with each of them independently, no process delays another. All this timing control and low-level specifications, however, make developing FPGA platforms difficult and time consuming.

The LabVIEW FPGA platform enabled me to program FPGAs without having to learn low-level hardware language. LabVIEW FPGA compiles code written in the standard LabVIEW interface into VHDL (Hardware Description Language). Although users require a thorough understanding of how to optimize FPGA code using fixed-point math, hand-shaking, and pipelining, for instance, they do not need to know the actual low-level syntax. LabVIEW FPGA has the additional benefit of a PC interface which I used to not only to save images and data, but also to monitor the experiment in real-time and manually control the stages when necessary. Although data is saved on the PC where rates are variable, I minimized timing uncertainty by bundling timestamps from the FPGA clock with the data. All GCaMP, tracking, and wide-field images are synchronized using these timestamps.

The algorithms used to process images and track the worm are simple and fast. In the $x, y, z$ tracking loop, the side-by-side, in and out of focus images of the marker neuron are first thresholded to remove all but the soma. The centroid of the soma from the in-
focus image is calculated to obtain x and y positions. The difference in power, sum of all pixel intensities, between the in and out of focus images is used as a measurement for z position. In the $\theta$ tracking loop, a different threshold is used to remove background noise but keep the cell process. The angular orientation of process is determined by first fitting an approximate spline. The process is first split into roughly 8 segments by binning pixel rows roughly perpendicular to the process, calculating the centroid in each of them, and then drawing a line between centroids from adjacent bins. The angular orientation of these segments are then averaged to obtain the process’ orientation. All of these position calculations occur as pixels are serially read from the camera and completed by the time the last pixel in the image is read. This way, the calculations do not add any latency and their speeds are limited only by the camera’s data transfer rate.
Figure 3.7: Real-time image processing and x,y,z, θ tracking on FPGA. (a) Raw dual-view image of marker neuron AWC imaged at two focal planes, z position: 1 and z position: 2. (b) The sharper and more in focus image from (a) is thresholded and splined to measure θ orientation. (c) Centroid position (x,y) is calculated from the sharper and more in focus image from (a). Z position is calculated from the difference in total intensity between the two z position images.

3.4.2. GCaMP Imaging

A liquid tunable lens placed in a relay system in the GCaMP imaging path allows z scan and volumetric imaging to be done without moving the objective lens and affecting tracking. I mimicked a configuration previously used for scanning through deep sections without any appreciable change in magnification or image quality\textsuperscript{112}. Rather than changing the objective to sample distance to vary the focal plane, a liquid lens with an adjustable focal length (Optotune EL-10-30-C) is used downstream in a relay system just
before the camera (Hamamatsu ImagEM). An FPGA (National Instruments PXIe-7961) is used to acquire images from the camera and control the liquid lens. Although higher resolution z sections would require a confocal, the high magnification and NA limits the depth of field to only 2 microns. Including the 30 millisecond exposure time, scan rates are 15.625 sections per second.

3.4.3. Structured Illumination

A DLP projector (Logic PD DLP LightCommander) was used to provide wide-field illumination for GCaMP imaging or spatially structured illumination or targeted photoactivation. A series of ~75mm relay lenses were used to map the image of the DLP to the sample plane and appropriate excitation filters were used depending on the application. The DLP was controlled using a combination of software written in LabVIEW and its native software. In my configuration, the DLP is limited to refreshing at 30 fps through HDMI but with more hardware level control, this could be increased to 60+ fps.

3.4.4. Image Analysis of GCaMP data

GCaMP information was extracted from the images by segmenting cells based on their probabilistic location in x,y, and z. Custom software was written in MATLAB to process images. A two dimensional histogram of the maximum intensity pixel locations for each frame is then generated. Regions where there are cells tend to have more maximum intensity pixels and therefore appear as peaks or clusters in the histogram (Figure 3.8b). The widths of these distributions is an estimate of how effective the
tracking is as better or worse tracking would decrease and increase the width, respectively. Most of the width is due to the rotation tracking not being able to correct for posture distortions during locomotion which is why the distributions resemble arcs that trace out larger angles the further they are from the marker neuron. Regions of interest were drawn by manually around each distribution and segmented as different neurons. In cases of z scans, the best focal plane is taken as that which maximizes intensity in the region of interest. The intensity of a neuron is taken to be the 95th percentile pixel intensity in the corresponding region of interest and at the best focal plane. The best focal plane was determined by tracking considering the probability distribution of the maximum pixel in each z stack (Figure 3.9).

Figure 3.8: Regions of interest and segmentation corresponding to different neurons in x,y are obtained from the maximum intensity projection. (a) Time series of z stack GCaMP images. (b) The maximum intensity projection of the time series of z stacks plotted as a heat map (a). Because of the image stabilization and tracking, each neuron stays localized to a specific regions of interest throughout the time series (shown by the regions circled in red). In this example, we were able to spatially resolve seven different regions of interest.
Figure 3.9: Neurons at different z positions in z-stack can be resolved by the maximum intensity. Within a region of interest that resolves neurons in x,y (Figure 3.8), neurons at different z position can be identified from the distribution of maximum intensity pixel containing slice each stack. (a) Time series of pixel of maximum intensity in each slice. The different z-stacks, taken sequentially in the time series are delimited by the dashed lines. Following the same principle as that described in the z-tracking, the intensity of a neuron increases towards the focused plane. In this example, there are two neurons at different z positions (annotated by black squares and open circles). (b) Histogram of the position within each z-stack containing the maximum intensity pixel over all z-stacks. The positions of the two peaks within this bimodal distribution are used to set z ranges, or regions of interest in z, for each of the two neurons. Slice position 1-8 would correspond to the black square, and 9-18 would correspond to the open circle.

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Chapter 4. Frequency Division Multiplexing Point

Detector Camera

Abstract

The latency of traditional Charge-Coupled Discharge (CCD) cameras is often limited by serial read-out. To address this, we designed and built a frequency division multiplexing single-pixel camera that transmits all pixel information simultaneously.

4.1. Introduction

Fast and sensitive cameras are used in numerous applications and are essential for tracking applications as described in Chapter 3. Although commercially available EMCCDs used in my microscope were sufficient to obtain the resolution and frame rates for my application, I asked if we could build a fast camera that overcomes some of their limitations. My goal was to image fluorescent neurons in a moving worm at millisecond timescales. One of the limitations of traditional CCDs is that the data from the pixels is digitalized and read serially. This may not be important in applications with low frame rates, but this latency limits high speed and real-time tracking. The latency could be reduced by reading pixel information in parallel instead of in series. To do this, I sought to encode space in the frequency domain, rather than the time domain.

Traditional EMCCDs digitize and read from spatially mapped pixels using time division multiplexing. The digitizer is essentially a point detector or channel which inherently has no spatial information. Each pixel and the region in space it corresponds to
is read in a particular known order, one after another. This technique has been used to make cameras from point detectors such as in laser scanning confocal microscopes.\textsuperscript{113,114} Time division multiplexing is what we typically do when having conversations with multiple people which we can refer to as channels—they take turns. We know from experience that this form of communication takes a long time. However, if the different people spoke at unique pitches we could distinguish different speakers and technically listen to them simultaneously. Whether our brains could simultaneously process multiple channels is a separate issue. This is an example of frequency division multiplexing which has been utilized by well over a century, most popularly in the form of AM radio. The idea behind frequency division multiplexing and the AM radio is to allocate different channels in frequency domain, technically referred to as bandwidth. Instead of getting chunks of time, users have different chunks of frequency. This is easily achieved by modulating each channel of some given bandwidth by a much faster and carrier frequency, hence the name \textit{Amplitude Modulation}.

I designed and built an optical interpretation of the AM radio by encoding different regions in space with frequency addresses. The main principles of the design are illustrated in Figure 4.1. Different regions in space, or pixels, blink at unique frequencies. If a fluorescent object is illuminated in one of those regions, it too will blink at the corresponding frequency provided that excitation frequency is slower than the typical fluorescence delay lifetime of several nanoseconds. The fluorescence emission light from all pixels can be collected onto a point detector after which a Fourier Transform can be used to pull out the frequency and corresponding region in space. In this example, a
frequency “B” is detected which corresponds to the [row: 1, column: 2] location in the 2x2 pixel camera.

I chose to use an avalanche photodiode as my detector because they are fast with excellent signal-to-noise. Avalanche photodiodes have a high bandwidth, typically 10+ GHz, and are ideal for low light applications because of their high sensitivity and low noise. Photomultiplier tubes which are commonly seen on commercial laser scanning microscopes could also be used but are typically much more expensive and were unnecessary for this study. Silicon avalanche photodiodes detect in the visible spectrum which was ideal for my fluorescence application. InGaAs avalanche photodiodes which detect in ~ 800-1700nm range offer the potential of infrared imaging which could be useful in astrophysics, for instance.

To generate the blinking pattern, I fabricated a high speed spatial light modulator that projects patterns from a spinning disk onto a sample. Commercially accessible DLPs do not have the speed and bandwidth needed for my high speed application and cost of acousto-optical tunable filters would outweigh their benefits. At that time, a group published a similar device that used a acousto-optic deflector combined with a acousto-optic frequency shifter to generate a 1D grid of frequency encoded pixels\textsuperscript{115}. There were several difficult challenges that I had to overcome in terms of fabricating, mounting, and stably spinning the patterned disk. Lastly, I tested the imaging system on a several fluorescent patterns.
Figure 4.1: Illustration of Frequency Division Multiplexing camera. Regions in space, spatially parsed into pixels, are unique encoded by different frequency addresses. The intensity illuminating each pixel blinks at a unique temporal frequency. When a fluorescence object is illuminated by one of these pixels, it emits fluorescent light that also fluctuates at the frequency of the pixel. By collecting emission light from all pixels and taking the Fourier Transform, it is possible to extract the spatial location of the object; all frequencies in the frequency spectrum that correspond to pixels covering the object will have non-zero amplitudes.
4.2. Results

4.2.1. Array of frequency encoded pixels were generated by intersecting sinewave patterns

To generate a pixel of array of different frequencies, I utilized the same idea used in amplitude modulation of modulating a signal frequency by a second carrier frequency. This idea is illustrated in Figure 4.2. Multiplication of two sinewaves produces two sinewaves with frequencies that are the difference and addition of the original sinewaves. In Figure 4.2, two lines with intensity patterns that are modulated as sinewaves are traveling to the left at a constant speed. This can be visualized as a light beam normal to the page shining through the pattern and at the reader. When viewed through a fixed window or slit, these two patterns generate intensity patterns that are modulated at frequency $f_A$ and $f_B$. If instead of a fixed window, the intensity is modulated by two other sinewave patterns of frequency $f_1$ and $f_2$, the resulting pattern is a 2x2 pixel array where the intensity in each pixel oscillates with frequencies $f_1 \pm f_A$, $f_2 \pm f_A$, $f_2 \pm f_B$, or $f_1 \pm f_B$. Intensity cannot be zero and all the intensity fluctuations are about some background or DC level. Therefore, all pixels in the same vertical column would also contain frequency $f_1$ or $f_2$, and all pixels in the same horizontal row would contain frequency $f_A$ or $f_B$. This can explained simply with a mathematical expression,

\[
(\text{DC}_A + \sin(f_A t)) \times (\text{DC}_1 + \sin(f_1 t)) = \text{DC}_A \text{DC}_1 + \text{DC}_A \sin(f_1 t) + \text{DC}_1 \sin(f_A t) + \frac{1}{2} \cos[(f_1 \pm f_A) t]
\]
Importantly, this means that because the minimum offset is equal to the amplitude of the sine, at least half the total power goes into frequencies by the same row or column (ACxDC terms), and at least a fourth goes into just DC (DCxDC term).

Figure 4.2: Generation of frequency encoded pixel array. A two dimensional pixel array of blinking patterns can be generated by intersecting travelling slits whose intensity or attenuation is spatially modulated as sinewaves. See Supplementary video.

4.2.2. Experimental Setup

Slits with sinewave intensity patterns were fabricated on a glass disk using photolithography. The fabrication and mounting of the disk, discussed in detail in the Materials and Methods, required overcoming numerous technical challenges with state-of-the-art lithography tools and new techniques. Twenty slower frequency slits (1-20kHz), corresponding to the signal channels, were illuminated with a uniform laser beam and the image of those slits was projected onto twenty faster frequency slits (40-800kHz),
corresponding to the carrier channels, using a series of relay lenses. The patterned disk, centered exactly along the motors axis of rotation, was spun at a high speed (100Hz) using a laser scanning air bearing motor to create the time varying blinking pattern described in the previous section. Slight imbalances and tilts could cause wobbles which at these high speeds could lead to dangerous and catastrophic failure. I therefore developed a protocol that was able to align the disk and motor rotation center to within a few microns. To minimize speed fluctuations, a custom closed-loop encoder system was built into the motor. The resulting light passing through both these patterns was then projected onto the sample plane using an objective lens. This frequency encoded 20x20 pixel array illuminates a fluorescent particle at the sample plane. The emitted fluorescent light was collected from the sample plan onto a point detector avalanche photodiode. The digitized intensity time series data was then passed to a Field Programmable Gate Array (FPGA) where an FFT was done and the frequency addresses, and hence the position, of the fluorescent particle was found. FPGAs where chosen over serial computers because in addition to having less computational overhead, they are more optimized for parallel processing which, when combined with the numerous parallel FFT algorithms such as Radix-2/4, makes them faster.
Figure 4.3: Experimental setup of FDM microscope. A 20x20 array of pixels each with its own unique frequency was generated by intersecting 1x20 patterns of sinewaves fabricated on a spinning disk. The fluorescence emission from a sample illuminated by the frequency encoded pixels was collected on an avalanche photodiode and the digitized intensity time series was then passed onto an FPGA where the FFT performed and the pixel addresses were identified.

4.2.3. Imaging

To demonstrate the feasibility of the FDM microscope, I imaged a sample of Rhodamine 6G particles roughly 10um in size with the intentions of eventually imaging live *C. elegans* neurons (Figure 4.4). The spectrum, though noisy, produced the expected results. In the frequency spectrum, the high speed carrier frequency combs were split by the lower frequency ones. The ACxDC terms also came through as expected. The
spinning disk was able to spin at a steady rate (<1% fluctuation in rotational speed) for at least tens of minutes (data not shown).

Figure 4.4: FDM microscope imaging 10um diameter spots. (a) Image of the rhodamine-6G sample taken with a commercial CCD. (b) Intensity of emission light

Figure 4.4: FDM microscope imaging 10um diameter spots. (a) Image of the rhodamine-6G sample taken with a commercial CCD. (b) Intensity of emission light
(Continued) collected over 5ms integration time. A Hamming apodization window function was applied to reduce spectral leakage in the DFT. (c) FFT of the time series from (b) computed in real-time on FPGA. Total computation time for a 16384 point FFT was <1ms. (d) Image of the sample reconstructed from the frequency spectrum in (b). Intensity of each pixel was taken to be the power in the spectrum at the corresponding frequency.

4.3. Discussion

I designed, built and demonstrated a 20x20 pixel frequency division multiplexing imaging system that could be used for fast imaging and overcomes the slow serial readout issue with traditional CCD cameras. The results were consistent with the original theory and, to the best of my knowledge, this is the only FDM imaging system that uses a mechanically moving pattern to modulate light intensity. The greatest challenges, mostly technical and involving the fabrication and mounting of the disk, were overcome using a variety of techniques and careful manipulation. Ultimately, the imaging system works but is not ideal for fluorescence applications due to its inefficient use of photons.

This FDM imaging system wastes light in the DC and ACxDC channels and is therefore not ideal for fluorescence applications in which every photon is important. The system only encodes and processes AC signals yet a DC is always present and not only increases photobleaching but adds background noise as well. This DC signal represents at least ¼ of the total light power. The ACxDC signals, although not containing unique pixel information, do contain some useful information. The presence of a signal at an ACxDC frequency implies that at least one pixel is illuminated along that particular row and column. The ACxDC frequency amplitudes, encode information similar to a “check sum”, where the amplitude or intensity is equal to the sum of the intensities of pixels in that row or column. Although useful, this check sum consumes at least ½ the total power.
The fact that light intensity is never negative and therefore always has a DC component means that using a purely AC processing scheme as is used in this FDM imaging system is not the most efficient.

Future work should include considering code division multiplexing (CDM) to encode space rather than FDM. In code division multiplexing, each channel is given a unique binary coding address. This is currently the most popular form of multiplexing in cellular phone use. To make an imaging system, each pixel in space would be coded by a string of on/off blinking events. In the 2x2 example from figure 4.1, the pixels could be given 01,00,10, and 11, for instance. The more unique each code is, which usually scales with length, the less crosstalk there is between channels. To once again draw upon the conversation analogy, a CDM conversation would be one in which you simultaneously listened to multiple speakers with the same pitch in voice, but who spoke in different languages.

4.4. Materials and Methods

4.4.1. Spinning disk fabrication, mounting, and leveling

Fabrication of the disk started with a high resolution (1um minimum feature size) chromium mask of pattern on quartz (Figure 4.5, 4.6). This was done by a commercial vendor using state-of-the-art photolithography. The center region of the mask was purposely left transparent for reason explained below. The starting size of the mask was 7” x 7” x 0.120”. Because this would have been too heavy to spin fast, I contracted a separate vendor to cut the square mask into a 125mm diameter disk and polish the
thickness down to 0.8 mm. A custom aluminum adapter was fabricated to attach the disk to the motor.

To align and mount the disk precisely to the rotation axis of the motor, I first determined the rotation axis using a camera. A dot was drawn on the face of the adapter and when spun at high speeds drew out a circle as imaged by the camera. The center of this circle was rotation axis. The disk was then aligned to rotation axis by first adding a layer of UV curable glue between the disk and adapter, positioning by carefully tapping the sides of the disk of hand, and finally curing the glue with a UV lamp. This is why the center of the disk was purposely left transparent. This centered the disk with the motor but tilt still had to be adjusted.

Even a small azimuthal between the rotation axis and disk would cause wobble when rotated. To minimize this, I added a tilt adjustment to the adapter. To quantify the wobble, I bounced a laser beam off the chromium surface and onto the wall several meters away. Without any azimuthal angle and wobble, the reflected laser spot on the wall would stay stationary as I rotated the disk. But because there was some wobble, the laser spot drew out an ellipse. I continually adjusted the tilt to minimize the ellipse and the final azimuthal angle was less than 0.05 degrees (Figure 4.7).
Figure 4.5: Schematic CAD of disk pattern. The disk pattern was created in MATLAB and saved as a .cif format. The pattern was then modified in a free software KLayout and saved as .gds. The pink shows regions that were transparent in the final disk.

Figure 4.6: High-magnification image of disk pattern. The sine wave pattern was created by varying the density of 1um chromium dots as sine waves. Shown here is a 100x magnification image of two of the slower (roughly 1kHz) patterns.
4.4.2. Setup

A green laser (Coherent Compass 115M-20mW 532nm) was used as the light source. The emission light was filtered through a high wavelength pass filter onto a TE cooled avalanche photodiode (Luna Optoelectronics SD197-70-74-661). The analog signal from the APD was then digitized and processed by an FPGA (National Instruments PXIe-766R, 5781 baseband transceiver) running custom written LabVIEW FPGA code.

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Chapter 5. Calcium Imaging and Targeted Photo-activation

Abstract

Using the tracking and image stabilization microscope developed in Chapter 3, I verified the neurons important for controlling speed (Chapter 2) individually with targeted photo-activation and calcium imaging with GCaMP. Calcium imaging revealed that some neurons exhibit fast second-scale dynamics whereas others show slow and long lived dynamics on the order of many minutes. These long lived dynamics, previously unmeasurable without our microscope, were correlated to behaviors with corresponding slow dynamics. SIA correlated with speed whereas RMG correlated with stop and go states.

5.1. Introduction

Measuring neuronal activity in freely moving animals enables us to correlate innate neuronal activity patterns with behavior. The dynamics of neuronal activity is commonly measured using the fluorescent calcium indicator, GCaMP. The tens of millisecond response time of GCaMP is suitable for imaging C. elegans because they do not produce action potentials, likely because they lack important Na,1 sodium channel required in organisms that do, but rather slower graded potentials\textsuperscript{29,30,85}. GCaMP dynamics for many of the neurons in the nematode’s nervous system have previously been measured in immobilized worms as well as in freely moving worms\textsuperscript{2,9,41,47,48,67,105}. These studies, most of which were focused on sensory neurons, have revealed that some
neurons sub-second responses to stimuli whereas others integrate over several seconds. However, as explained in Chapter 3, all of these studies have been limited to measuring dynamics on timescales of up to only a few minutes. It is therefore unknown if there are neurons with slow dynamics and if they drive behaviors such as local-global search in chemotaxis and speed modulation. However, our microscope now allows us to probe these longer timescales.

Using the tracking and image stabilization microscope, I verified that individually inhibiting the key neurons identified in the Archaerhodopsin behavioral screen (Chapter 2), RMG, SIA, and AVB, is sufficient to elicit the slowing down phenotype and measured their dynamics in freely moving animals. AVB is already known to control speed\textsuperscript{47,51,59} so I focused my attention on the lesser studied neurons RMG and SIA. As a control, I first imaged GCaMP in two well-studied neurons BAG and AIY and compared my results to those measured from microfluidics devices or other tracking and imaging systems. BAG responded to odor with the same second-scale response as found in previous studies\textsuperscript{116}. Surprisingly, although previously characterized as an odor sensing neuron, it also showed burst-like activity without any odor stimuli present. These activities were correlated with the animal’s touching itself, a novel observation that could not have previously been made using constrained animals in microfluidic devices. I also found that AIY exhibited slow dynamics and long lived states that correlated with reversal frequency—not individual reversal events as found in previous studies\textsuperscript{49}. I then imaged GCaMP in SIA and RMG and found that the first correlated well with all fluctuations in speed whereas the latter correlated to stop and go states which typically lasting several minutes.
5.2. Results

5.2.1. Targeted inhibition of key neuron RMG

I inhibited the interneuron RMG using spatially patterned illumination in a freely moving worm to verify that it was sufficient to produce the slowing down speed phenotype observed in the \textit{flp-21::arch} line from the Arch screen (Figure 5.1). As shown in Chapter 3, our microscope is capable of selectively illuminating cells less than 20 \textmu m apart in a moving animal. Inhibiting other neurons in the promoter such as URA and the M2 neuron, however, did not affect speed (data not shown). Therefore, RMG is sufficient and likely necessary for the speed phenotype observed when all \textit{flp-21} expressing neurons are inhibited.
Figure 5.1: Targeted inhibition of RMG alone is sufficient to elicit speed phenotype.
(a) Speed of a nematode in which RMG is periodically inhibited by the targeted illumination (grey). Worms slowed down significantly when RMG was inhibited. (b) RMG inhibited animals showed significant slowing down compared to control animals (not fed all-trans retinal). N denotes the number of individual worms and trials. Error bars show standard deviation.
5.2.2. *Calcium imaging reveals neurons with fast or slow dynamics*

For comparison, I measured calcium dynamics in two neurons BAG and AIY which have previously been measured in microfluidics devices or other tracking systems. BAG activity, as measured using GCaMP, is known to respond positively to liquid OP50 bacteria supernatant in a microfluidics device\(^9\). I gently delivered OP50 odors onto the worm and observed similar responses in terms of fluorescent fold change and second timescales (Figure 5.2a). Interestingly, I also observed frequent and significant activity even without the presence of odor (Figure 5.2b). BAG activity increased when the animal touched itself, typically during reversals, and also spontaneously without any touching. This observation would not have been possible in a constrained animal and suggests a novel role for BAG in mechanical touch response.

![Figure 5.2: BAG calcium activity in freely moving worms.](image)

(a) BAG responds positively to OP50 odors (grey bars). (b) Calcium dynamics of BAG without any odor stimulant. Grey bars denote events where the animal touches itself, typically during a reversal. Open circles activity not correlated with touch.
The interneuron AIY is known to be involved in controlling reversals\textsuperscript{39,40,87,117}. Tonic inhibition of AIY increases reverse rates and GCaMP imaging using other methods have shown it to be correlated to reversal events on a minute timescale\textsuperscript{49}. Using our microscope, I imaged GCaMP in AIY for up to an hour and found slow dynamics with tens-of-minutes timescales (Figure 5.3). These slow dynamics in AIY have not previously been reported. For animals that are transferred off food and imaged, the AIY activity level is usually initially low, but after 15 to 20 minutes increases and stays at a higher level for at least tens of minutes. These two states, as shown by the bimodal distribution of AIY levels (Figure 5.3b), correlate with the well-studied local-global search transition where animals in the local search state reverse more frequently in time and space (Figure 5.3c).
Figure 5.3: AIY Calcium activity in freely moving worms transferred off-food shows long lived states correlated to reversal rate and local-global search. (a) Upon removal from food, AIY activity is low for ~15-20 minutes after which it goes high. Time axis shows time after removal from food. The underlined regions denote artifacts due to errors in the microscope’s tracking. (b) Histogram of the AIY activity time series in (a) shows bimodality which is consistent with there being two AIY states: high and low. (c) Trajectory of the animal with its AIY activity from (a) overlaid in color.
5.2.3. *Calcium dynamics in SIA, RMG correlate with speed*

Having shown that calcium dynamics in some neurons exhibit slow dynamics, I asked if such dynamics in SIA and RMG controlled the slower minute scale modulations in speed (Figure 1.1, 2.8c). We could measure the effect of driving fast or slow dynamics by continuously modulate arch at different frequencies, but it was easier to directly measure GCaMP activity. I measured GCaMP activity in SIA, RMG, AIY and URA while simultaneously measuring speed (Figure 5.4a-d). To measure correlations between GCaMP activity and speed on slower timescales, I first filtered out higher frequency components using a moving average smoothing window and then calculated the Pearson correlation (Figure 5.4e-h). I did this for smoothing windows up to 4 minutes long, about 1/10th the length of a typical time series, with the longer smoothing windows retaining only slower frequencies. The speed to GCaMP activity correlations for both RMG and SIA improved significantly when 30 second and faster frequency components which likely included head-lifts and other neck movements were filtered out. The correlations continued to improve at longer smoothing windows but at a slower rate.

On average, SIA activity correlated well with speed but surprisingly, most RMG traces showed only weakly positive correlations with the exception of a few which were strong (Figure 5.4e-f). The Pearson correlation between SIA and speed was greater than 0.5 for smoothing windows larger than 1 minute and statistically larger than zero over all smoothing windows. The two out of the seven RMG traces that were most strongly correlated with speed were qualitatively different in that they showed stopping events where the animal would slow to less than a few microns per second for several minutes at a time. Stopping was correlated with a decrease in RMG activity, but smaller speed
fluctuations during forward movement were not (Figure 5.4b). As a negative control, I correlated speed with GCaMP activity in neurons that, from the literature and Arch behavioral screen, were not expected to control speed. URA and AIY exhibited dynamics on different timescales, URA showed fast minute scale spiking similar to BAG, but as expected neither correlated with speed (Figure 5.4c-d, g-h). URA had a coefficient comparable to SIA and was a false positive in the L2-norm solution (Figure 2.10b).

Figure 5.4: SIA, RMG, AIY, URA calcium dynamics correlated with speed. (a)-(d) Time series of GCaMP activity (blue) and speed (orange). Typical time series were are 30-50 minutes long. The time series in (d) corresponds to the purple curve in (f). (e)-(f)
(Continued) Pearson correlation of GCaMP activity and speed (a-d) as a function of the smoothing window size. Different colors represent different animals and trials. Region bounded by mean plus and minus standard deviation are shown in grey.

5.3. Discussion

Using our microscope, I imaged GCaMP activity in freely moving worms for up to an hour and observed slow dynamics which were previously not possible to measure. Imaging at high temporal resolution for many minutes allowed me to correlate activity with short event such as reversal and self-touch as well as longer lived behavior such as local-global search. In the case of BAG and AIY, I found that the activity correlated with self-touch and reversal rate, respectively. Even though GCaMP activity in these neurons has been previously been recorded, measurements using our microscope system has revealed novel phenotypes.

Consistent with the results from the behavioral screen and sparse solution, GCaMP activity in SIA and RMG correlated with speed but the strength of correlation for the latter depended on whether or not the animal paused during the experiment. SIA was correlated to speed on all timescales and intensity levels. RMG, however, showed slower dynamics and was correlated to stop and go. It is possible that SIA acts more as a fine analog control, whereas RMG is more of a discrete on-off switch for locomotion. SIA is post-synaptic to RMG which suggests that the higher frequency activity pattern in SIA is not driven by RMG but by a different neuron upstream or self-generated. The two neurons might be part of two separate modules for controlling speed. This also demonstrates how the connectivity map alone cannot predict how signals are propagated.

The behavioral screen, sparse solution, and GCaMP imaging uncovered important neurons around which to look. Having found neurons whose dynamics controls speed, we
can look upstream and ask which neurons, if any, also have such dynamics. The hope is that we can use this approach to find the neuron or circuit that generates the pattern and where decisions are made. SIA is post-synaptic to only a handful of neurons, including RMG, and pre-synaptic to none. RMG does not share the same dynamics which leaves only 8 candidate upstream neurons: RIV, URB, CEP, URX, ALM, ADE, RIA, RIB, AVK. The same can be done with RMG, although there are more than two dozen possible upstream neurons.

Because we can do targeted inhibition using spatially patterned illumination, as demonstrated using RMG, we can also do electrophysiology in a moving animal. By photo-activating Arch or ChR2 in neurons and measuring GCaMP downstream, we can infer functional connectivity\textsuperscript{47}. It would be interesting to see if shutting down RMG also shuts down SIA in which case the latter may operate as a frequency division multiplexing receiver that integrates slow signals from RMG and faster ones from a different neuron, or channel. Similarly, it is interesting that RMG anatomically resembles a “hub interneuron” and is post-synaptic or gap-junctioned with several sensory neurons such as ASH, HSN, and AWB which show fast second-scale dynamics yet RMG shows a lower bandwidth\textsuperscript{47,118,119}. It is possible that RMG acts as a low pass filter in which it integrates all signals over long timescales, or the coupling strength is weaker between it and fast timescale neurons. Bandwidth is an important topic in characterizing computer circuits and communications networks and our microscope now provides a platform with which we can start to address these kinds of questions in small nervous systems\textsuperscript{120–123}. 

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5.4. Materials and Methods

5.4.1. Strains

Strains were grown and maintained under standard conditions unless indicated otherwise. Transgenic lines with pha-1 selection marker were grown under 24°C. All experiments were done in lite-1 mutants to minimize the animal's sensitivity to blue light.

5.4.2. Odor delivery

Room air (30 SCCM) was bubbled through saturated OP50 bacterial liquid culture in a 50mL Falcon tube before being gently delivered to the worm (~1 cm away from exit nozzle). Similarly, a separate air supply was bubbled through tube of distilled water to act as a “no-odor” condition. An electric three-way valve (The Lee Company) was used to switch between the two odor conditions with sub-second resolution.
Chapter 6. Discussion

I developed a framework to uncover essential neurons for behaviors in small nervous systems and measure their dynamics. These tools have revealed activity patterns such as slow dynamics in several neurons that have not been reported and provide a platform with which we can start to address fundamental questions about signal processing and context dependence in nervous systems.

6.1. Summary

Understanding how information flows and decisions are made in nervous systems is a difficult problem. If we think that every neuron and all the connections between them are important, the space of parameters we need to consider becomes astronomically large. If we were given a computer circuit board and wanted to understand its architecture and how computations were done, where would we begin? The traditional approach in neuroscience has been to break the complex problem into simpler ones. Instead of looking at the entire circuit board, probe the function of smaller sub-circuits one at a time. While obviously not the same as a computer circuit board, the nervous system of *C. elegans* has several important characteristics that make it a model organism for such an approach and understanding small nervous systems. Its small nervous system of only 302 neurons is anatomically invariant and the connectome was mapped more than thirty years ago. Knowing the anatomical connections, however, is not the whole story. Although the functions of neurons can be roughly classified based on the presence of anatomical structures such as neuromuscular junctions\textsuperscript{44}, we cannot predict how information flows through and the functional architecture of this high interconnected network. Perturbations
of a sparse number of neurons in *C. elegans* have shown that not all neurons are essential for behaviors\(^36,39,59,60,126,127\). It would seem that we do not need to study the entire nervous to understand a specific behavior, but rather just focus on the important neurons and circuits.

I addressed several challenges related to uncovering essential neurons and measuring their dynamics. Perturbing neurons one by one using laser ablation has revealed several neurons essential for behaviors such as reversals, aggregation, and egg-laying, but with compressive sensing and optogenetics, I was able to sample nearly the entire nervous system using 41 lines and uncover a sparse solution which I then verified to be consistent. I showed using simulations that for a sparse set of essential neurons, perturbing multiple neurons at a time and minimizing the L1-norm to find the sparse solution is more efficient. Because many promoters are expressed in multiple neurons\(^43\), I used them to my advantage and drove the expression of photoactivatable Archaerhodopsin-3 to inhibit specific subset of the nervous system. I applied this method to screen for neurons essential in controlling speed and found SIA, RMG, and AVB, the latter two which were expected based on the literature. To study their dynamics, I designed and built an image stabilization microscope that allowed me to measure GCaMP activity with high spatio-temporal resolution and also enabled me to do targeted inhibition of individual neurons. Not only were the GCaMP activities in these neurons correlated with speed and consistent with the behavioral screen results, I also observed neuronal dynamics on the order of tens of minutes that have not been reported.

Uncovering a few essential neurons for function and measuring their dynamics is not sufficient to understand the nervous system. There are clearly more than three
neurons involved in controlling speed and this could change depending on context and the internal state of the worm. This framework allows us find functionally important neurons which combined with the anatomical map can help us discover circuits. Assuming continuity, one would want to look at both pre-synaptic and post-synaptic neurons. Furthermore, the observation that neurons show dynamics on different timescales raises important questions about bandwidth and how neurons, most of which resemble integration hubs\textsuperscript{29}, process multiple inputs. How do we reconcile connections between neurons with calcium activities on different timescales? Fundamental questions such as how dynamics are generated on different timescales and the role of bandwidth in the nematode’s nervous system still need to be addressed. If we make the naïve simplification that the connections between the neurons are wires and that processes are not bandwidth limited, the small and highly interconnected nervous system would resemble a complex mess of short circuits and redundant pathways. How do sub-circuits even arise and are there multiple circuits controlling the same behavior? If so, does the importance or utilization of the circuits change which would suggest that there is context dependent signal interpretation? Signaling molecules and neurotransmitters would be useful to study since they play a role in shaping the functional connectivity and dynamics\textsuperscript{42}. Although we initially treated the nervous system as fixed and rigid, there is plasticity and function may be indeterministic. Perhaps this is what most differentiates a live nervous system from a computer circuit.
6.2. Slow dynamics and bandwidth

Our microscope allows us to probe neuronal activity on the timescale of an hour. Previous imaging systems have been limited to observing neuronal activity and behaviors up to a few minutes \(^3,^9,^{49,95,97,98}\). Functional mapping has therefore been based on these short correlations, which is probably why reversal events are one of the most studied behaviors. Several important and well-studied behaviors such as the local-global search transition during chemotaxis happen on a timescale of tens of minutes and using our microscope we found that it was correlated to AIY activity. Being able to look for longer gives us a better chance of studying states and their transitions. In the example of RMG (Figure 5.4b), the correlation between calcium dynamics and speed depended on the stop and go state of the worm. A good way to study context dependent signal interpretation is to see it in the same worm. Otherwise, how do we know if variability in the dynamics is due to worm to worm variability, or context dependence, or both? We currently do not know how slow dynamics are generated and the role of frequency dependence in signal processing.

Frequency response and bandwidth are generally important in signal processing\(^{128,129}\). If a neuron integrates signals, then it has a finite frequency response and bandwidth. One might expect that fast frequency components are continually filtered out as they are integrated by downstream neurons. How then are the sharp spikes driving reversal events generated, for instance those such as those in the command interneuron AVA\(^{130}\)? Are there separate pathways for propagating and processing fast versus slow signals? Perhaps neurons and their activity patterns, although anatomically connected, are not all functionally connected because of what is effectively bandwidth mismatch.
Different signal channels may be encoded in frequency space as described in Chapter 4. We can start asking these kinds of questions and using our microscope we can measure the bandwidth and integration time of downstream neurons by comparing the frequency spectrum with upstream neurons or by directly driving upstream neurons at different frequencies using optogenetics. Different circuit elements may be tuned to different frequencies and perhaps this influences which behaviors they control. Knowing the bandwidth of different neurons may also point us to where memory is stored.

6.3. Signaling molecules and context dependence

Although *C. elegans* has a small nervous system, it has a surprisingly diverse neuronal genome. For instance, the number of neuropeptides is roughly equal to the number of neurons and significantly more G-protein-coupled and acetylcholine receptors than humans. Functional connectivity is shaped by neuromodulators that can modify the dynamics, excitability, and synaptic function. Dopamine, glutamate, and neuropeptide PDF are involved in local/global search and their dynamics may be what underlie the slow calcium activity dynamics I observed. The interneuron AIY whose activity correlates with local/global search has receptors for all of these neurotransmitters. It is possible that this diversity is what allows the anatomically small nervous system to perform complex computations and helps contextualize the circuit. Octanol avoidance is mediated by ASH in well-fed animals, but after starvation is distributed between ASH, AWB, and ADL, and this transition can be recapitulated using exogenous serotonin. The important neurons that I found controlling speed were in the context of well-fed worms performing chemotaxis and may be different for starved...
worms. In tracking and imaging systems that use dangerously high light levels, the context might be of a damaged or injured worm. One interpretation is that the anatomical wiring of the worm tells us potential pathways, but that at any one time there is only a subset of accessible paths as defined by the internal state of the neuromodulators.

Instead of first screening for important neurons and then determining the relevant signaling molecules, one can start by asking which of the latter control behavior, and then which neurons secrete or receive these signals. Receptor expression has traditionally been done catalogued by driving fluorescence proteins under promoters for the receptors. Recent advances in single-cell RNA sequencing, however, have made it easier to determine which cells and express receptors for and synthesize various signaling molecules\(^\text{134–137}\). Combining these tools with our framework may allow us to construct a more complete picture for small nervous system, from the systems level down to the molecular details.

6.4. Conclusion

In trying to understand a nervous system, as with \textit{C. elegans}, the static wiring diagram is a good place to start. Over the past few decades, it has guided numerous functional experiments and led to the discovery of essential neurons and circuits, both important aspects of the architecture. This map, and our understanding of nervous systems, is increasing not only in detail, but dimension are well—time and neuromodulator states are dimensions, for instance. We are now at the stage where we can look beyond what the computational units are and start observing them and probing fundamental questions of signal processing in nervous systems.
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**Appendix: Supplementary Movie of X,Y,Z,θ tracking and image stabilization**

Supplementary movie titled “Supplementary Movie S1.mp4” illustrates the challenges of tracking and stabilizing the movement of freely moving nematodes at high magnification as described in Chapter 3. The movie was taken on our developed microscope and shows performance without tracking, with xy, with xyz, and with xyz θ tracking. Playback speed is roughly 1.5x.