FAST IMAGING REVEALS HOW EXPERIENCE MODIFIES NEURAL STRUCTURE AND FUNCTION IN AN AWAKE BRAIN

by

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Abstract

During learning, neurons in the brain are remodelled in both their structure and function in ways that can dramatically shape how they process incoming information and compute an output. This is especially the case in early development when neurons are extremely plastic. The spatial arrangement of a neuron’s synapses determines how inputs interact to perform computations, such as through recruitment of nonlinear conductances by spatially clustered activity. However, it remains poorly understood how such functional arrangements arise. Here, I generate the capabilities that allow me to record and analyze sensory-evoked activity in an in vivo model system and to analyze how sensory inputs shape neural activity. I develop and validate the genetic and optical tools and protocols that allow me to record neural activity across the neuron, being able to capture both synaptic input across the dendritic arbor and the action potential output. Leveraging the albino Xenopus laevis visual system as an accessible vertebrate model of early brain circuit formation I then use these tools to image growing neurons during plasticity-inducing visual training while recording and manipulating neuronal firing. I show that dendrite growth and pruning are correlated to neurons’ evoked calcium responses. Lastly, I use the functional data from these experiments to build and validate a mathematical model to predict locations of synaptic input on dendritic arbors, using fluorescence-based calcium data.
Lay Summary

Developing neurons are extremely dynamic and their rapid changes in both structure and activity during development significantly shape how they compute information. Our understanding of this developmental plasticity is currently incomplete. Here, I develop the tools and technologies to be able to record the complete array of activity across a single neuron as well as tracking its structural and functional changes. I use these novel capabilities to be able to perform experiments that demonstrate how sensory input produces patterned changes in neural structure and function as well as generating and validating a predictive model of synaptic input on a dendritic arbor.
Preface

The work in Chapter 3 has previously been published:


Dr. Kelly Sakaki designed and built the imaging platform published in this paper and programmed the user interface. Dr. Kaspar Podgorski built a previous prototype fast-scanning microscope that served as a basis for the redesigned version. I assisted with building the imaging platform, modified the optical train and performed all of the in vivo validation experiments. Patrick Coleman improved the user interface (UI) and added in the Segmented Scanning function. Dr. Kelly Sakaki and I both wrote the manuscript and prepared the figures. Professor Kurt Haas provided advice on platform and experimental design and assisted in preparing the text.

The work in Chapter 5 has previously been published:


Dr. Claire Guerrier and I contributed equally to this work. Dr. Claire Guerrier and Nicolas Galtier designed the model and performed the virtual simulations. I performed the in vivo experiments
and analyzed the experimental data. Dr. Claire Guerrier and I both wrote the text of manuscript. Nicolas Galtier prepared the figures. Professor Kurt Haas assisted in preparing the text.

All studies were performed with the approval of UBC Animal Care Committee and were in accordance with the Canadian Council on Animal Care (CCAC) guidelines. Animal Care Number: A190297
# Table of Contents

Abstract ........................................................................................................................................ iii
Lay Summary ................................................................................................................................. iv
Preface ........................................................................................................................................... v
Table of Contents ........................................................................................................................ v
List of Tables ................................................................................................................................. vii
List of Figures ............................................................................................................................... xi
List of Abbreviations ..................................................................................................................... xiv
Acknowledgments ......................................................................................................................... xviii
Dedication ...................................................................................................................................... xix

Chapter 1: Introduction .................................................................................................................. 1

1.1 Synaptic Plasticity and learning on a cellular level ................................................................. 1
   1.1.1 Plasticity at a synapse ........................................................................................................ 1
   1.1.2 Theoretical models of plasticity and meta-plasticity ....................................................... 3
   1.1.3 The existence and role of clustering of synaptic inputs ................................................... 4

1.2 Fast imaging in in vivo systems ............................................................................................. 6
   1.2.1 Two-photon scanning laser microscopy .......................................................................... 6
   1.2.2 Fast point-scanning utilizing acousto-optics ................................................................. 8

1.3 Recording and interpreting neural activity in vivo ............................................................... 10
   1.3.1 Using calcium as a marker for activity .......................................................................... 10
   1.3.2 Deconvolving calcium signals to extrapolate activity ................................................... 11
   1.3.3 Modeling neural activity .............................................................................................. 13
   1.3.4 In vivo imaging of glutamate inputs in neurons ............................................................ 18

1.4 Investigating neural plasticity in a developing brain ............................................................. 20
   1.4.1 Xenopus tadpoles as an ideal model for investigating neural encoding ......................... 21
   1.4.2 Research Aims .............................................................................................................. 22

Chapter 2: Fast in vivo imaging across the dendritic arbor ....................................................... 23

2.1 Introduction ........................................................................................................................... 23

2.2 Designing a customized calcium sensor .............................................................................. 24

2.3 Methods ............................................................................................................................... 27
   2.3.1 Plasmid design .............................................................................................................. 28
   2.3.2 Expression of plasmid constructs .................................................................................. 28
Chapter 3: Manuscript 1 - Comprehensive imaging of sensory-evoked activity of entire neurons within the awake developing brain using ultrafast AOD-based random-access two-photon microscopy

3.1 Preface: Fast sampling of neural activity in an in vivo model ............................................................... 30
3.2 Summary .................................................................................................................................................. 32
3.3 Introduction ............................................................................................................................................. 33
3.4 Methods ................................................................................................................................................ 39
  3.4.1 AOD-based random-access microscope optical train ................................................................. 39
  3.4.2 Piezo-actuator and POI-based trajectory ....................................................................................... 41
  3.4.3 AODs and high speed POI scanning ............................................................................................... 43
  3.4.4 POI Scan ........................................................................................................................................... 44
  3.4.5 AOD POI laser deflection programming ....................................................................................... 44
  3.4.6 Piezo-actuator and AOD Synchronization ..................................................................................... 45
  3.4.7 System requirements ....................................................................................................................... 47
  3.4.8 Temporal sampling considerations ................................................................................................. 47
  3.4.9 Spatial sampling considerations ..................................................................................................... 50
  3.4.10 Temporal and spatial scanning limitations .................................................................................... 51
  3.4.11 Microscope driver software design ............................................................................................... 51
  3.4.12 Graphical User Interfaces ............................................................................................................. 52
  3.4.13 Proposed event sequence for comprehensive imaging .............................................................. 54
  3.4.14 Rapid scanning scheme ................................................................................................................ 57
  3.4.15 System validation .......................................................................................................................... 57
  3.4.16 Xenopus laevis preparation ........................................................................................................... 59
  3.4.17 Animal stimulation chamber with visual stimulator ................................................................. 60
3.5 Results .................................................................................................................................................. 63
  3.5.1 Validating the optical train ............................................................................................................. 63
  3.5.2 Piezo-actuator validation ................................................................................................................. 64
  3.5.3 Rapid-scan position validation ....................................................................................................... 65
  3.5.4 Experimental validation and 4D data collection ........................................................................... 65
3.6 Discussion ........................................................................................................................................... 74

Chapter 4: Sensory experience modifies developing neurons ................................................................. 78
  4.1 Introduction: Investigating neural plasticity in an in vivo developing model ..................................... 78
  4.2 Results .................................................................................................................................................. 81
4.2.1 Tectal neurons can be separated into groups based on activity and the different activity groups have distinct response patterns to plasticity inducing visual stimuli ....... 82
4.2.2 Spaced training modifies dendritic arbor growth and remodelling in a context sensitive pattern .................................................................................. 87
4.2.3 Spaced training modifies filopodial survival and responsiveness ...................... 91
4.2.4 Potentiation of action potential output is linked to potentiation of synaptic inputs 101
4.2.5 Glutamatergic input is linked to patterned structural plasticity ....................... 110
4.3 Discussion ........................................................................................................ 119
4.4 Summary of findings .......................................................................................... 124
4.5 Methods ............................................................................................................. 127
4.5.1 Animal rearing conditions: .............................................................................. 127
4.5.2 Expression of genetically-encoded fluorophores: .......................................... 127
4.5.3 Target neurons .................................................................................................. 128
4.5.4 In vivo imaging of single neuron activity: ....................................................... 128
4.5.5 Visual stimulation and plasticity inducing protocol: ....................................... 128
4.5.6 Processing of calcium imaging data ................................................................. 130
4.5.7 Grouping of neurons by activity and plasticity ............................................... 131
4.5.8 Spatial clustering .............................................................................................. 132
4.5.9 Statistics ........................................................................................................... 132

Chapter 5: Manuscript 2- An algorithm based on a Cable-Nernst Planck model predicting synaptic activity throughout the dendritic arbor with micron specificity .................. 133

5.1 Preface: Designing and validating a model of synaptic driven calcium flux in an in vivo model system ................................................................. 133
5.2 Summary ............................................................................................................. 133
5.3 Introduction ........................................................................................................ 135
5.4 Methods ............................................................................................................. 138
5.4.1 Experimental protocol .................................................................................... 138
5.4.2 Mathematical modeling .................................................................................. 140
5.4.3 Data analysis .................................................................................................. 145
5.5 Results ............................................................................................................... 146
5.6 Discussion ......................................................................................................... 156
5.7 Conclusion ......................................................................................................... 163

Chapter 6: Conclusion .......................................................................................... 165
6.1 Summary of Aims .............................................................................................. 165
6.2 Strengths, limitations and future directions ............................................................ 165

Bibliography ....................................................................................................................... 170
List of Tables

Table 5-1 Simulation parameters for the channels dynamics ........................................ 164
List of Figures

Figure 2-1 Testing the function of a P2A self-cleaving peptide in Xenopus tadpoles .......... 27
Figure 3-1 Comprehensive imaging ................................................................. 37
Figure 3-2 Optical train and the stimulation module .............................................. 40
Figure 3-3 POI distribution and Z-actuator schedule and trajectory ......................... 42
Figure 3-4 Timing diagram for system synchronization ...................................... 46
Figure 3-5 Visual-evoked calcium responses in a brain neuron using GCaMP6m .... 49
Figure 3-6 System user interface ........................................................................ 53
Figure 3-7 Scan class hierarchy and scan types .................................................. 55
Figure 3-8 The experiment protocol used to validate the microscope ...................... 58
Figure 3-9 The visual stimulation chamber ......................................................... 62
Figure 3-10 Optical train and piezoactuator performance validation ....................... 64
Figure 3-11 Visually evoked tectal neural activity can be recorded across the complete dendritic arbor and soma using random access TPLSM .......................................................... 69
Figure 3-12 Visually evoked synaptic calcium transients across the neuron can be recorded with increased temporal resolution using Segmented Scanning ........................................ 71
Figure 3-13 Visually evoked synaptic-localized glutamate transients across the neuron can be detected using Segmented Scanning ................................................................. 73
Figure 4-1 In vivo recording of changes in structure and function of tectal neurons ...... 81
Figure 4-2 Neuron maturation state does not predict responsivity or plasticity in response to spaced training ........................................................................................................ 84
Figure 4-3 Action potential activity correlates with dendritic activity .................... 86
Figure 4-4 Plasticity in activity is linked to patterns of structural changes ............... 88
Figure 4-5 Spaced training drives clustered additions and distributed subtractions ... 91
Figure 4-6 Clustering of filopodia added during spaced training to existing responsive filopodia promotes survival ................................................................. 94
Figure 4-7 Filopodial additions arising during spaced training that are clustered to existing OFF responsive filopodia are more likely to be themselves responsive to OFF ........................................ 96
Figure 4-8 Filopodial additions arising during training that are responsive are more likely to survive .................................................................................................................. 98
Figure 4-9 Dendrite growth behavior directed by local calcium transients promotes clustering of tuned synapses ................................................................. 98
Figure 4-10 Intracellular distance from OFF responding synapses predicts survival of additions that arise during spaced training ................................................................. 100
Figure 4-11 Figure 4-11 Potentiation of output is correlated with potentiation of input ... 103
Figure 4-12 Stimulus evoked calcium transients can be detected across the dendritic arbor and spatially isolated ................................................................. 103
Figure 4-13 Potentiation of output is correlated with increased synaptic strength in additions post-training ................................................................. 107
Figure 4-14 Potentiation of output is correlated with pruning of less responsive filopodia ... 109
Figure 4-15 Glutamatergic input modifies filopodial growth and survival ................ 114
Figure 4-16 Glutamatergic inputs are not globally modified in response to spaced training .... 116
Figure 4-17 Spaced training strengthens synapses both presynaptically and postsynaptically ... 119
Figure 4-18 Synaptic input promotes patterned remodeling .................................... 125
Figure 4-19 In vivo spaced training experiment protocol ..................................... 126
Figure 5-1 A one-dimensional model of a three-dimensional dendritic arbor......................... 147
Figure 5-2 Comparison between the coupled and the decoupled model .............................. 149
Figure 5-3 Simulation of voltage, calcium and jGCaMP7s-Ca dynamics in a dendritic arbor  . 150
Figure 5-4 The rising slopes of stimulus-evoked calcium transients indicate the location of active synapses in simulation and experimental data ........................................................................... 155
Figure 5-5 Comparison between experimental and simulated calcium traces ...................... 158
List of Abbreviations

A1 – Primary auditory cortex

ABS – Artola, Brocher and Singer Rule

AF – Actor framework

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA – Analysis of variance

AOD – Acousto-optic deflector

AP – Action potential

BCM – Bienenstock-Cooper-Munro theory

Ca2+ – Calcium

CamKII – Calcium–calmodulin dependent protein kinase II

CAG – cytomegalovirus early enhancer/chicken β-actin

CCAC – Canadian Council on Animal Care

DYNAMO – Dynamic morphometrics

ED – Extracellular distance
EGFP – enhance green fluorescent protein

F3DS – Full 3D scan

FRET – Fluorescence Resonance Energy Transfer

FFT – Fast Fourier transform

FWHM – Full width at half maximum

GABA – γ-aminobutyric acid receptor

GECI – Genetically-encoded calcium indicators

Glt1 – Glutamate transporter 1

HEPES – Hydroxyethylpiperazine Ethane Sulfonic Acid

ID – Intracellular distance

iGluSnFR – intensity-based glutamate-sensing fluorescent reporter

LTD – Long term depression

LTP – Long-term potentiation

LVOOP – LabVIEW Object-Oriented Programming

Mg2+ – Magnesium
mCyRFP1 – monomeric cyan-excitable red fluorescent protein 1

Na+ – Sodium

NCX – Na+/Ca2+ exchangers

NIR – Near infrared

NMDA – N-methyl D-aspartate

NND – Nearest neighbour distance

PDMS – Polydimethylsiloxane

PLA – Polyactic acid

POI – Points of interest

PMT – Photomultiplier tubes

RAMP – Random access multiphoton

RC – Resistor capacitor circuit

RGC – Retinal ganglion cell

RS – Rapid scan

RFP – Red fluorescent protein
RIPA – Radioimmunoprecipitation assay buffer

SCE – Single cell electroporation

SDS PAGE – Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

SEM – Standard error of mean

sfGFP – Superfolder green fluorescent protein

SLAP – Scanned line angular projection

STP – Short-term potentiation

TDBL – Total dendritic branch length

TPLSM – two-photon scanning laser microscopy

UI – User interface

V1 – Primary visual cortex

VGCC – Voltage-gated calcium channels

WBE – Whole brain electroporation
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Dedication

To my wife, Aliya who makes all of this have meaning and value. I cannot wait to start the next chapter of our lives together.
Chapter 1: Introduction

1.1 Synaptic Plasticity and learning on a cellular level

1.1.1 Plasticity at a synapse

The dendritic arbors of growing neurons in the developing brain are extremely dynamic, with a rapid turnover of filopodial protrusions, which are associated with synapse formation, strengthening and loss (El-Boustani et al., 2018; Haas et al., 2006; Hossain et al., 2012; Sin et al., 2002). While there is evidence for the role of non-activity dependent mechanisms driving synaptogenesis, with a complete loss of neurotransmitter release nonetheless allowing for the formation of synapses in a developing brain (Verhage et al., 2000), subsequent research supports a role for activity in sculpting dendritic structure and synaptic organization. Overall arbor growth and complexity of neurons in vivo are strongly linked to synapse formation (Niell et al., 2004) and this growth is mediated in an activity-dependent manner (Espinosa et al., 2009; Haas et al., 2006). These synaptic activity-driven changes in dendritic arbor remodeling are dependent on glutamatergic transmission through both AMPA- (Haas et al., 2006) and NMDA-subtypes of glutamatergic receptor-mediated activity (Espinosa et al., 2009; Sin et al., 2002). The presence of AMPARs is necessary for activity-induced dendritic arbor growth, synapse stabilization and synapse maturation (Haas et al., 2006). NMDAR activity has been shown to be necessary for visually-induced total dendritic arbor growth and increased rates of branch additions as well as for the maturation of synapses located on new dendritic filopodia (Li et al., 2011; Sin et al., 2002) and for the stabilization of the axonal branch inputs that innervate these tectal neurons (Kutsarova et al., 2016; Munz et al., 2014).
There is also substantial data establishing a link between these same biochemical pathways and experience-dependent modulation of neural activity. The classic model is built around the ability of NMDARs for coincidence detection of pre- and post-synaptic activity, where synchronous presynaptic glutamatergic release and post-synaptic depolarization is required for NMDAR activation via removal of Mg$^{2+}$ blockade, allowing influx of calcium that activates downstream plasticity pathways (Jedlicka, 2002). Sufficient postsynaptic depolarization to induce unblocking of NMDARs can be achieved by strong synaptic input(s) or be driven by back-propagating action potentials (Bi & Poo, 1998; Jedlicka, 2002; Linden, 1999; Stuart & Sakmann, 1994). While the coincidence detection ability of NMDARs is the primary driver of the induction of an increase in synaptic strength, or ‘long-term potentiation’ (LTP), AMPARs have been shown to play a key role in LTP maintenance (Jedlicka, 2002; Kandel E.R., Schwartz J.H., Jessel T.M, 2000). Modification of AMPAR phosphorylation, expression level at synapses (T. Takahashi et al., 2003; Tao & Poo, 2001) and subunit composition (Adesnik & Nicoll, 2007; Derkach et al., 1999; H.-K. Lee et al., 2000) have been shown to mediate the maintenance of synaptic strength. Reversible changes in the phosphorylation of specific intracellular residues of AMPARs such as phosphorylation of Serine 831 on GluA1 by CamKII resulting in increased single-channel conductance (Derkach et al., 1999) have been demonstrated to be a key modulator of the maintenance of synaptic strength (H.-K. Lee et al., 2000). Longer duration maintenance of LTP is attributed to an increase in number of AMPARs at the synaptic membrane, while activity-driven weakening of synaptic strength, or long-term depression (LTD) is associated with reduced AMPAR number (Tao & Poo, 2001).
1.1.2 Theoretical models of plasticity and meta-plasticity

Several models were proposed in the 20th century to explain and predict activity driven changes in synaptic strength. An early and still influential model is Hebbian plasticity (Hebb, 1949), which posits, “neurons that fire together will wire together”, meaning that there would be a causal relationship between repeated pre-synaptic input and post-synaptic strength. However, one shortcoming with the Hebbian model is that it struggles to explain LTD and furthermore, if neural plasticity were to be driven entirely by Hebbian mechanisms you would see all neurons being inexorably driven along one of two opposing paths towards the extremes of either runaway activity or quiescence (Turrigiano & Nelson, 2004). As such, there is a need for a second form of plasticity that works opposed to Hebbian plasticity, keeping neurons in a state of homeostasis. A second, later theory that builds on the Hebbian model and works to address this is called Bienenstock-Cooper-Munro (BCM) theory (Bienenstock et al., 1982). BCM theory makes two main claims: first, that a neuron possesses a synaptic modification threshold, and that whether or not its activity is above or below this threshold at any given instant will determine whether the neurons inputs will strengthen, leading to LTP or weaken leading to LTD. The second claim is that this plasticity threshold is itself plastic (i.e. metaplastic) and that consequently, the amount of activity input necessary for a neuron to switch its response from LTD to LTP can change over time. This allows for the role of homeostatic plasticity to be accounted for with the activity threshold for LTP being a function of previous synaptic activity and increases in activity consequently leading to an increase in the threshold for future input to cause further LTP. BCM theory has been supported by in vivo experiments measuring evoked neuronal somatic responses corresponding to action potential firing whereby exposure to visual white-noise stimulus
significantly shifted the probability of a neuron potentiating to a subsequent plasticity-inducing stimulus (Dunfield & Haas, 2009).

Related to BCM theory, the Artola, Brocher and Singer (ABS) Rule accounts for heterosynaptic plasticity (Artola & Singer, 1993). As in the BCM model, there is a threshold for activity, above which the neuron will undergo LTP and below which it will undergo LTD. This LTD/LTP threshold is entirely based on the membrane potential, and hence, the intracellular calcium concentration of the post-synaptic neuron. As a result of this, LTP or LTD induction can occur not just at active synapses, but also at neighbouring synapses. It is posited that these synapses need not be active themselves as local calcium influx from nearby active synapses is sufficient to depolarize the membrane at these synapses (Artola & Singer, 1993; Jedlicka, 2002). Therefore, ABS theory provides an explanation for not just the modification of synaptic strength of an individual active synapse, but also for surrounding clustered synapses.

1.1.3 The existence and role of clustering of synaptic inputs

The bulk of historical research on synaptic plasticity has employed non-physiological electrical stimulation of large afferent input populations and population recordings from 100s-1000s of neurons in ex vivo brain slice preparations. However, technological advances now allow investigations of physiologic, sensory-driven plasticity of individual neurons within intact and awake brains. Furthermore, there is concerted effort aimed at understanding how individual neurons compute information, including how the organization of synaptic inputs onto the dendritic arbor contributes to signal integration (Kerlin et al., 2019; Makino & Malinow, 2011; Scholl et al., 2017). In experiments where sensory input drove synaptic potentiation at individual synapses, it was found that neighbouring synapses showed a reduced threshold for potentiation, demonstrating
spatial aspects of functional plasticity across dendrites (Harvey & Svoboda, 2007). Further, research has demonstrated that stimulus-driven synaptic potentiation in dendrites occurs in clusters of synapses on short stretches of dendrites in mouse barrel cortex neurons, as indicated by an enrichment of GluR1-containing AMPA receptors at these synapses (Makino & Malinow, 2011). This clustered pattern of potentiation was eliminated when the mice were deprived of sensory experience by having their whiskers cut, or when a mutant AMPAR was expressed that was insensitive to plasticity-associated protein kinases (Makino & Malinow, 2011).

There is ample evidence for a patterned synaptic topography at the microcircuit level (N. Takahashi, 2019). Neighbouring neurons (Markram et al., 1997; Song et al., 2005), as well as neurons encoding similar stimuli (Ohki et al., 2005) have been shown to have a higher than chance number of reciprocal afferent connections between them, forming spatially and functionally distinct clusters of connected neurons. There is also anatomical evidence that individual axons form multiple synapses onto dendrites in a spatially localized pattern, potentially allowing for clustered synchronous synaptic inputs on dendrites (Bloss et al., 2018; Schmidt et al., 2017). Such topographical clustering of synapses driven by the same upstream inputs is thought to be key to modulating how neurons integrate information in their dendritic arbors. If present, a spatial clustering of coincidently firing inputs would allow for nonlinear amplification of their contribution to post-synaptic potentials, resulting in a far stronger dendritic signal then the linear summation of co-active synapses at distant sites across the dendritic arbor (Losonczy et al., 2008; Polsky et al., 2004).

Recent in vivo studies report evidence for spatial clustering of inputs. In neurons in primary visual cortex, V1, in ferrets adjacent synapses on dendritic branches tended to preferentially respond to similarly oriented bars (Scholl et al., 2017; Wilson et al., 2016). Similar results were
found in neurons in mouse V1, where dendritic spines with similar receptive fields tended to be spatially clustered (Iacaruso et al., 2017). However, other groups investigating synaptic topography in vivo in mouse V1 (Jia et al., 2010) and primary auditory cortex A1 (X. Chen et al., 2011) found that synapses that encode similar stimuli were randomly dispersed throughout dendrites, with no evidence of spatial clustering. The highly variable findings in this research area leaves the question of the role and even the existence of clustering of synapses based on their tuning in vivo. A substantial caveat to all these in vivo studies is that in each case, they are only recording stimulus-driven activity across a subset of synapses a small section of dendrite, leaving the investigation of global synaptic topography across whole dendritic arbors as yet unexplored.

A further currently unaddressed area of study is how clustering of synapses based on tuning can arise during early brain circuit formation when synapses and dendrites are formed, as most research analyzing established circuits has focused on mature animals (Kerlin et al., 2019; Makino & Malinow, 2011; Scholl et al., 2017).

1.2 Fast imaging in in vivo systems

1.2.1 Two-photon scanning laser microscopy

To be able to generate an all-or-nothing axonal output, neurons receive and integrate numerous dendritic inputs across their 3-dimensional arbor structure. Thus, to be able to investigate how experience shapes dendritic growth, synaptogenesis and receptive field development, a major challenge resides in efficiently scanning and analyzing the activity of the complete arbor of individual neurons in vivo. While high-resolution analysis of calcium activity in cultured neurons is relatively
straightforward using fast wide-field or confocal imaging (H.-K. Lee et al., 2000), attempting to perform similar experiments of complex 3D neural structures in conscious vertebrates required volume scanning at velocities sufficient to track changes in calcium dynamics, on the order of tens to hundreds of milliseconds.

Through utilizing Near-Infrared Laser (NIR) scanning, conventional two-photon scanning laser microscopy (TPLSM) makes it feasible to image at near millimetre depths allowing for in vivo imaging of dendritic arbors in brain tissue that would be impossible with wide-field or confocal microscopy, (Denk et al., 1994; Svoboda et al., 1997). Typically, the scanning laser is controlled in the x and y-axis by a set of galvometric mirrors. This method of laser control has the benefit of flexibility in control of the laser scanning space. The user is able to either scan across the complete field of view through employing numerous sequences of line-scans (i.e., “rastering”) to image a single 2D X-Y image-plane. Alternatively, galvometric mirrors can scan subsections of the field of view or perform individual line-scans. However, regardless of the type of scanning, the design of galvometric mirror-based systems has a fundamental problem that limits its capability to achieve efficient scanning at high speeds. This is that their scanning efficiency is limited by inertia-induced latencies as the mirrors have to change direction during x and y-axis transitions. When these directional transitions are minimal, such as when scanning a single line on a single plane, which requires a single axis transition, fast sampling rates near 100Hz can be achieved. However, when trying to raster across a 2D space to generate an image, where there are numerous X-, and Y-axis transitions as the laser rasters multiple lines, the sampling rate drops precipitously. As a result, 512 x 512 images on a single plane can result in scan rates of only a few Hz. An alternative form of mirror based-guidance of a scanning laser is resonant scanning, in which the mirrors controlling x and y-axis scanning of the laser beam oscillate at a continuous rate. This can
allow scanning the same 512 x 512 image on the order of tens of Hz, with the trade-off of lacking the flexibility due to the resonant scanning motor being driven by a single frequency.

While both resonant scanning over a complete 2D field of view or galvo-based scanning over a limited number of rastering lines can record at rates sufficient for recording neural activity across a dendritic arbor, this scan rate drops precipitously when attempting to scan a 3D space, such as scanning across a dendritic arbor. Even ignoring the additional time necessary to change the depth of the focal point of the imaging system in between raster-scanning each x-y plane in order to scan a 3D volume, scanning a volume of 100 μm x 100 μm x 100 μm with a step size of 1 μm means even a resonant scanning based system capable of sampling a plane at 20Hz would only be able to sample the complete volume at a rate of no more then one scan every 5 seconds. This scanning rate would make it impossible to sample neural activity using fluorescence based calcium sensors.

### 1.2.2 Fast point-scanning utilizing acousto-optics

One potential way to increase the scanning speed of a neural arbor is to reduce the number of points sampled per plane. The neuron itself, being primarily composed of long, thin and branching dendrites takes up only a small fraction of the space on each image plane, and thus the vast majority of the points being sampled during a raster scan are of unlabeled tissue. Consequently, utilizing a scanning method that allows for sampling only the comparatively small amount of space on each imaging plane occupied by the neuron itself offers the potential of huge gains in sampling rate. This has lead to the recent development of “Random Access Multi-Photon (RAMP) Microscopy” (Bullen et al., 1997; Katona et al., 2012). RAMP microscopes achieve faster sampling rates in 3D volumes through eliminating plane-by-plane raster scanning. Instead, RAMP platforms only sample pre-determined Points of Interest (POIs) without sampling the space between these points, greatly reducing
the number of points being sampled and thus potentially increasing the sampling rate. RAMP microscopes have recently been applied to recording calcium activity in the dendritic arbor (Kerlin et al., 2019).

To perform random access sampling, galvometric mirrors are replaced with acousto-optic deflectors (AODs) to direct the laser beam. Unlike mirrors, which reflect an incoming laser beam and are driven by a motor that repositions the angle of the mirror to change the position of the laser beam, AODs are composed of crystals that refract the incoming laser beam. The key property of AODs is that this refraction index can be modified by passing sound waves at different frequencies through the crystal. Consequently, by driving a pair of AODs with a piezo motor, it is possible to create a laser positioning system that is capable of scanning across an X-Y plane. Crucially, since there are no physical components to constantly reposition, as is the case with mirrors, this is an inertia free scanning system. As a result, platforms utilizing AODs can record points of interest on a plane at kHz-rate sampling rate (Bullen et al., 1997; Bullen & Saggau, 1999; Iyer et al., 2003; Salomé et al., 2006).

Since the sampling rate of AODs is directly related to the number of points sampled on a plane, regardless of the position of these points across the field of view, unlike mirrors, where changing the direction of the rotation of the mirror represents a major time-loss, AODs are ideal for sampling a limited number of randomly distributed points across a plane at an extremely fast sampling rate, rather than rastering across a full field of view at a slower rate. This fast sampling rate across a limited number of points of interest can be leveraged in order to record using fluorescence biosensors such as voltage sensors which are so rapid, that kilohertz sampling rates are required (Villette et al., 2019). However, this capability is also ideal for sampling a dendritic arbor, where the total amount of space occupied by the labeled neuron on each individual plane is only a small fraction of the total space. As
such, acousto-optic deflector based RAMP imaging systems have the potential to be the ideal tool for fast in vivo imaging of the complete structure of an individual neuron.

1.3 Recording and interpreting neural activity in vivo

1.3.1 Using calcium as a marker for activity

Calcium is a common second messenger in many types of cells, regulating cellular metabolism, cytoskeletal remodelling, exocytosis, gene transcription, and programmed cell death and hence intracellular calcium concentrations are highly regulated (Brini et al., 2014). Neurons tightly control the calcium gradient across their membranes with intracellular concentrations being on the order of 10000 times lower than extracellular levels (Gleichmann & Mattson, 2011). This concentration gradient is maintained by various membrane channels, chiefly ATPase pumps and exchangers, mainly Na\(^+\)/Ca\(^{2+}\) exchangers (NCXs) (Brini et al., 2014).

Calcium imaging is an established proxy for measuring neural activity (T. Chen et al., 2013; Nakai et al., 2001). Rapid spikes in intracellular calcium correlate strongly with the depolarization at the cell membrane associated with synaptic input and action potentials (T. Chen et al., 2013; Gleichmann & Mattson, 2011; Nakai et al., 2001). Through employing calcium-sensitive fluorescent dyes or genetically-encoded calcium indicators (GECIs) this activity can be visualized through fluorescence based microscopy (Nakai et al., 2001; Rose et al., 2014). Using calcium as a proxy for neural activity has the advantage of being a method capable of easily labeling and visualizing action potentials from a large number of neurons in vivo, opening the door for analyzing whole neural circuits (Yang & Yuste, 2017). Additionally, through the use of techniques such as single-cell electroporation (Haas et al., 2001; Hewapathirane & Haas, 2008)
calcium can be used to visualize activity across the neural structure (El-Boustani et al., 2018; Sakaki et al., 2020). Recently developed generations of Genetically-Encoded Calcium Indicators (GECIs), such as the jGCaMP7s, further refine the kinetics of calcium sensors which compared to other GECIs, boasts an unparalleled sensitivity and dynamic range (Dana et al., 2019). However, the kinetics of calcium and its indicators do not directly equate to the underlying voltage, with both fluorescence based recordings of action potential and synaptically mediated calcium influxes being far slower than the underlying voltage changes due to their calcium binding kinetics (T. Chen et al., 2013; Katona et al., 2012; Nakai et al., 2001; Sun et al., 2013). This slower rate of dynamic change when recording calcium transients using fluorescence-based calcium sensors can be vital for recording neuronal activity because it means that the temporal recording requirement to achieve Nyquist sampling is far less than it would to use a fluorescence based-voltage sensor. However, using fluorescence based calcium sensors has necessitated the creation of modelling and deconvolution systems to distinguish the types and sources of calcium transients in neurons.

1.3.2 Deconvolving calcium signals to extrapolate activity

To be able to take fluorescence based calcium recordings across a neural structure and from them extract and interpret the underlying neural activity it is necessary to be able to understand the calcium dynamics that correlate with different types of activity, from the synaptic inputs, to action potential output. This is because there is a wide variety of voltage and ligand gated calcium channels across the surface of the neuron that are involved in input and output functions. At synapses, NMDA and certain AMPA subtypes of glutamate receptors drive calcium influx (Burnashev et al., 1992), and these transients can trigger the opening of nearby voltage-gated calcium channels (Cowan et al., 2001; Oertner & Svoboda, 2002; Sabatini et al., 2002; Sabatini &
Svoboda, 2000). The depolarization during an action potential also triggers more calcium channel opening near the soma and it is through calcium flux driven by these somatic channels that we commonly infer the presence of action potentials in fluorescence based experiments (T. Chen et al., 2013; Gleichmann & Mattson, 2011; Nakai et al., 2001).

In neurons, calcium transients reported by fluorescence based sensors demonstrate fast rise times and slow exponential decays (T. Chen et al., 2013; Deneux et al., 2016; Friedrich et al., 2017; Podgorski & Haas, 2013). While these slow decays can be beneficial for event detection using imaging platforms sampling at slower rates, they also can lead to multiple events blending together into a continuous plateau of signal, when underlying electrophysiological truth is that there is a series of rapid but discrete events. In response to this, there have been multiple attempts to design algorithms to deconvolve activity from changes in raw calcium fluorescence (Friedrich et al., 2017; Podgorski & Haas, 2013; Sakaki et al., 2018; Shen et al., 2022; Vogelstein et al., 2010). Different approaches include non-negative deconvolution (NND) (Podgorski & Haas, 2013; Vogelstein et al., 2010), which infers the most likely spike train based on the assumption spikes must be positive, and thus the data can be filtered by this. Other methods include different fitting techniques, matching the recorded data to a template of what the fluorescence of a single spike should look like and then “peeling” off individual spikes from the data until a signal can be deconvolved (Grewe et al., 2010). Recently, there have been attempts to deconvolve calcium data by creating models of both intracellular calcium dynamics and the baseline fluorescence of the cell, and by comparing the real world data, attempt to predict the most likely spike train underlying the measured calcium fluorescence (Deneux et al., 2016). Modelled systems have a set of parameters that are fed into the model, such as different estimated fluorescent amplitudes and decay times for single spike that can be modified by the user to adapt the model for different types
of neurons, adding to the versatility of model-based deconvolution systems. Models also tend to be modular, allowing for the incorporation of new physiological details allowing for continued improvement of performance. Meta-analysis between estimated and true spike data from simulated data shows that different deconvolution methods yield generally acceptable results, but that the number of estimated spikes might vary somewhat across methods and that when these algorithms are applied across a group of neurons to infer population encoding, this can lead to a different distribution of estimated firing rates and estimated numbers of tuned neurons (Evans et al., 2019). Thus, it remains an ongoing challenge in the field to create a universal deconvolution system for fluorescence based calcium activity.

1.3.3 Modeling neural activity

Due to their slow kinetics and the impressive array of specialized and refined dyes and genetically encoded sensors, fluorescence based calcium sensors are often the standard for trying to experimentally record activity across a neural structure. In contrast to direct electrophysiological recordings through patch-clamp, these sensors are much less invasive and additionally make tracking activity in multiple locations of a neuron and in small compartments like filopodia or dendritic spines much more feasible. However, beyond solely looking at calcium fluxes, there have been numerous attempts extrapolate the broader underlying ionic fluxes and voltage dynamics across a neuron.

The membrane potential of a neuron is the major physical variable that enables information processing in a neuron. It has three attributes that make it so. First, it is able to function at a fast rate, maintaining and utilizing an electrochemical gradient between the internal and external side of the membrane, allowing for a rapid change in membrane potential to be triggered through the
opening of various ion channels. Secondly, this diverse array of channels allows the membrane to precisely modify its voltage in a vast array of different ways, allowing it to implement a variety of linear and nonlinear operations. Lastly, these channels allow the membrane potential to represent inputs from the physical world, encoding sensory input and then driving an output in the form of neurotransmitters (Keyes, 1985; Koch, 2004). As a result, there have been multiple models created with the goal to characterize voltage dynamics across the neural membrane.

The simplest model of a neural membrane is a basic resistor-capacitor (RC) circuit (Koch, 2004). In this basic model the neuron is represented as a single spherical compartment. This hypothetical spherical neuron has an electrical potential across the membrane, with a negative voltage internally maintained by a dynamic equilibrium of ions across the membrane, defined as the resting potential ($V_{rest}$). This resting potential is actively maintained by the neuron with roughly half the energy used by the brain being devoted to the maintenance of this equilibrium (Ames et al., 1992; Koch, 2004). While there is controlled flow of ions across the membrane through protein channels, the phospholipid bilayer acts as a thin insulator separating the more negatively charged cytoplasm from the extracellular medium. This thin insulator separating two differently charged conducting solutions acts as a capacitor ($C$) with this capacitance proportional to the area of the membrane. When the voltage applied across the capacitance changes, there is a capacitance current as the amount of charge on either side of the plasma membrane changes. There is no direct flow of charge directly across the membrane, but rather a redistribution of charges along either side. What endows this membrane to be dynamic and hence encode information is the proteins embedded in it. These proteins serve as receptors, channels and pumps allowing the controlled flow of ions across the membrane. In effect, they can be described as resistors in the RC circuit and they determine the membrane resistance ($R_m$). If you were to assume this system
was passive and you were to drive a current into this neuron, you would see the neuron act as a shift-invariant linear system, with the resultant membrane potential showing a corresponding shift. This can be thought of being akin to pumping air into a leaky balloon (the balloon is the membrane, the embedded protein ion channels are the holes), where the inflow of air (the injected current) would stretch the balloon (the flexibility would be akin to the capacitance) until a new equilibrium was found. Synaptic input can then be built into the model. Excitatory post synaptic potentials (EPSPs) and inhibitory post synaptic potentials can be added as localized, transient changes in the conductance across the membrane. The conductance change for a synapse will be dependent on what type of ions it allows across the membrane. This will determine the synaptic reversal potential ($E_{\text{syn}}$), of a synapse, which if it is greater then $V_{\text{rest}}$ will result in an inward, depolarizing flow of current and if $E_{\text{syn}}$ is lesser than $V_{\text{rest}}$ you have an outward, hyperpolarizing flow of current. Thus, it is possible to model both excitatory and inhibitory input into a neuron.

Neurons however, are not spheres, so the next stage in modeling a neuron is to incorporate a more realistic shape to the neural structure, that of a series of cylinders. From this, we get “Cable theory”. Cable theory was originally based on the study of how electrical signals propagate along mechanical cables where there is a conductive core surrounded by an insulator (Koch, 2004). Cable theory was then applied to modelling neural activity alongside experiments working with the axons of giant squids (Davis & Lorente de No, 1947; Hodgkin & Rushton, 1946) and subsequently applied to dendrites (Rall, 1989). With neurons, the cell membrane takes the place of the insulating layer of the cable and the cytoplasm is the conductive core. Because of the insulating casing of the neuronal membrane and its elongated cable shape, you get an almost exclusively longitudinally directional flow of current down the cable, as opposed to the case of the basic RC model sphere. Similar to electrical wires, the passive resistance will be dependent on the
radius of the cable, with a thin radius dendritic segment having more resistance then a thick one. Hence, there is an inverse relationship between the passive $R_m$ and the circumference of a dendritic segment. There is also an inverse relationship between cable radius and intracellular resistance ($R_i$). Conversely, there is a positive relationship between membrane capacitance ($C_m$) and membrane radius because the larger the cable the more membrane there is to be able to store a charge. The increase in $C_m$ and decrease in $R_m$ are forces that act to reduce the propagation of charge, such as that caused by a synaptic input, down a larger dendrite, as it leads to more charge staying localized to the membrane or leaking across the membrane. However, the decrease in $R_i$ leads to an increase in the propagation of an input charge, and this factor outweighs the changes in $C_m$ and $R_m$ because as the radius of the neuron increases the internal volume, which determines $R_i$ increases at a greater rate (volume is a function of $r^2$) than the membrane area which determines $C_m$ and $R_m$. Hence, the propagation of a current generated from a synaptic input can be modeled through dendrites of variable diameter. When scaled up to a complete neuron modelled as a collection of tubular segments, this means that smaller neurons require less charge in order to reach depolarization threshold. This is because due to their small axon and soma sizes, they have a lower $C_m$ and higher $R_m$. This leads to the “Size principle” when applied to motor units whereby smaller motor neurons are recruited first and larger motor neurons are recruited last (Gordon et al., 2004; Henneman et al., 1965).

However, in actuality neuron membranes are not passive resistors with resistances directly based on their volume. This is because ion flow across the membrane is tightly controlled by a broad array of ion channels. The Hodgkin-Huxley model has been the most widely used model for modelling action potential initiation and propagation in a neuron. This model takes the basic electric circuit model where the cell membrane is modelled as a capacitor separating ionic charges
and adds ion channels as resistors allowing charge to cross the membrane. The main channels in this model are voltage gated sodium, potassium and leak channels (Hodgkin & Huxley, 1952). For sodium and potassium channels, the current of each is based on the difference between the membrane potential \((V_m)\) and the Nernst potential (ie. the voltage needed to maintain an unequal concentration of a particular ion across a membrane) and the conductivity of each ion across the membrane, which is also voltage dependent (since the sodium and potassium channels are voltage gated). The leak current is likewise dependent on the difference between \(V_m\) and the composite Nernst potential of all the different ion species that flow through leak channels and the conductivity. However, the conductivity of ion leak is not dependent on voltage since these channels are not voltage gated. The total current across the membrane is the sum currents of sodium, potassium and the various ions through the leak channels. Each of the sodium and potassium channels has a probability of being open or closed and can cycle between either state based on \(V_m\) and time. The fraction of channels that are in an open vs closed state determines conductance and it is the change in \(V_m\) which determines the rate at which closed channels open vs open channels close (termed the “Gating variable”), and thus overall conductance (Hodgkin & Huxley, 1952). Thus, it is possible to model at what voltages populations of sodium and potassium channels will collectively be predominantly open or closed and recapitulate the dynamics of an action potential. Hodgkin-Huxley formalism has since been extended to modelling ion flow in dendrites and other models have added Hodgkin-Huxley type channels for calcium current (Guerrier & Holcman, 2017; Petousakis et al., 2022).

While cable theory and Hodgkin-Huxley modelling are instrumental tools in building the basis for many neuron models, specific models can become significantly complex, because the biological system they are representing is vastly sophisticated. There is a large array of different
ion channels with different conductances that are distributed in different concentrations across different neural compartments. Channels can be ligand gated, voltage gated or coincidence detectors like NMDARs and to add to that, synaptic inputs can change conductance values non-linearly (Gonzalez et al., 2022; Lavzin et al., 2012; Redmond & Ghosh, 2005). Furthermore, the fine detail of neural structure can be immensely complex and shape the flow of ions through the region of the arbor, with the whole models being designed to spine neck (Lagache et al., 2019). Thus, as our understanding of the intricacies of how neurons process information, new models are continuously being generated to characterize the underlying voltage dynamics and to address how neurons act as individual computational units.

1.3.4 *In vivo* imaging of glutamate inputs in neurons

Neurons receive hundreds or even thousands of inputs and integrate this information, which allows them to perform complex input-output computations. Emerging evidence demonstrates that the spatial arrangement of these synaptic inputs on the dendritic arbor could play an essential role in these computations, allowing for non-linear signal amplification (Gonzalez et al., 2022; Lavzin et al., 2012; Redmond & Ghosh, 2005). However, specifics of how the layout and structure dendritic arbor and distribution of the synapses on it contribute to integration of synaptic information remains unknown. Today, it is in increasingly feasible to record synaptic activity in individual neurons either directly through fluctuations in membrane charge using dendritic patch-clamp electrophysiology or through using calcium as a proxy-measurement through employing genetically encoded fluorescent calcium indicators (Basak & Narayanan, 2018; Dana et al., 2019; London & Häusser, 2005). While patch clamping offers unparalleled temporal resolution of neuronal voltage transients, fluorescence based calcium indicators have several key advantages
that make them the preferred tool for certain applications such as being non-invasive and, if the imaging platform is capable of it, providing a recording of activity across the complete neuronal structure.

However, relying on calcium transients as a proxy measurement for synaptic input has limitations. While calcium influx into neurons is correlated with membrane voltage changes driven by either action potentials or synaptic inputs the relationship between the two is not linear, with calcium dynamics being orders of magnitude slower than membrane voltage changes which can result in difficulty deconvolving rapid bursting activity. Furthermore, since there are multiple sources of calcium input, including channels at the synapse, various voltage gated calcium channels driven by back-propagating calcium channels and the release from internal stores such as the endoplasmic reticulum, it is a substantial challenge to distinguish the different sources of calcium (Guerrier et al., 2022). Lastly, while calcium activity can be a feasible proxy for how a neuron responds to synaptic input, it does not provide direct information about the characteristics of the actual synaptic input at a neuron. This has led to the need to develop fluorescence based sensors that directly record neurotransmitter input. These sensors include glutamate, acetylcholine, GABA, norepinephrine, dopamine and glycine (Leopold et al., 2019).

As the primary excitatory neurotransmitter in the vertebrate central nervous system, it is of key importance to characterize the input of glutamate. Synaptic glutamatergic transmission is essential for a wide array of neural functions including such as learning and memory, long-term potentiation and synaptic plasticity. Extrasynaptic glutamatergic drives cell death and dysregulation of glutamatergic activity is linked to multiple neurodegenerative diseases including Parkinson's, Alzheimer's and Huntington's diseases (Marvin et al., 2013; Willard & Koochekpour, 2013). The most developed of the fluorescence-based neurotransmitter sensors is the glutamate
sensor, iGluSnFR, which has undergone several rounds of improvement (Marvin et al., 2013, 2018). Early versions of iGluSnFR proved to be sufficiently sensitive to be capable of discriminating stimulus evoked glutamate release in both cultured neural tissue and across several model organisms including worms, zebrafish and mice (Marvin et al., 2013). The second generation of glutamate sensors improved the sensor’s functional brightness and stability replacing the original circularly permuted EGFP with a circularly permuted superfolder GFP, while also designing multiple variants that either emphasized sensitivity or speed (Marvin et al., 2018). Despite these improvements, discriminating the specific targets of glutamate release remained challenging. This was due to these sensors exhibiting saturating activation kinetics. Furthermore, the receptor itself was excluded from post-synaptic densities. Thus, while the second-generation iGluSnFR variants were capable of detecting discreet, spatially isolated glutamate release events, it was difficult to ascertain whether they were synaptic or extrasynaptic in origin.

The most recent improvements to iGluSnFR specifically aim to address this deficit (Aggarwal et al., 2022). This new version has non-saturating activation kinetics and are faster then the previous versions, allowing for better discrimination of glutamate release events based on their kinetics. Furthermore, these new sensors have been modified to overcome the synaptic exclusion of previous iGluSnFR constructs with one version fused to a fragment of stargazin, a protein that has been demonstrated to regulate AMPA receptor targeting to synapses (Cuadra et al., 2004). These improvements to iGluSnFR along with improvements in fast in vivo imaging enable new possibilities for discriminating synaptic glutamate from either spill over or extrasynaptic glutamate based on fluorescence responses.

1.4 Investigating neural plasticity in a developing brain
1.4.1 *Xenopus* tadpoles as an ideal model for investigating neural encoding

Neurons are the fundamental units of computation in all advanced organisms. Thus, understanding how a neuron encodes information through both its unique structure and functional arrangement of its inputs is an essential question at the core of our understanding of cognition. Furthermore, we know that as an organism develops and learns, these structural and functional arrangements are in flux. This means that to properly comprehend how neurons compute information and how this underlies an organism’s learning and cognition, it is essential to use an *in vivo* model and to analyze encoding in a neuron, not just at a single timepoint, but also across a time period, while the animal is receiving some sort of information input.

To that end, the visual system of the albino *Xenopus laevis* tadpole represents an exceptional experimental model. *Xenopus* has the advantage of being small and transparent, allowing for easy access to the brain for both fluorescently labeling neurons and subsequently imaging them. The visual processing system of albino *Xenopus* tadpoles, the optic tectum is easily accessible, with only transparent skin covering the brain rather than a skull and, the neurons themselves are relatively small, with the complete dendritic arbor and the soma being able to be visualized in a 100 μm x 100 μm x 100 μm imaging area. This means that the complete structure of a tectal neuron is potentially accessible using two-photon microscopy. In addition, of key importance the animal can be kept conscious and immobilized resulting in minimal motion artifacts. This is in contrast to *in vivo* brain imaging in rodents, where motion artifacts are a major challenge to overcome and require specialized techniques and technologies to compensate (Leinweber et al., 2014; W. Liu et al., 2022; Paukert & Bergles, 2012). Since *Xenopus* tadpoles presented an easily accessible experimental system, with a comparatively small neural volume and
minimal motion artifacts, this opens up the potential to sample the complete neural structure *in vivo* of an animal receiving stimulus, in the form of patterns of light and darkness.

1.4.2 Research Aims

My doctoral work was focussed on three related objectives: Firstly, to design the tools and methods that would enable me to record the whole repertoire of activity across the complete structure of a single neuron. Secondly, to then apply these tools to be able to characterize experience-induced tuning patterns across the dendritic arbor and their impact on structural growth of neurons in the intact and awake developing brain. Lastly, I wanted to utilize this experimental dataset to be able to build and validate a model of a single neuron, creating a tool that would allow for improved analysis of fluorescence-based functional datasets. The advantages of the *Xenopus* tadpole model synergizes with the strengths and limitations of RAMP microscopy, with the small arbor volume and minimal motion artifacts inherent in neural imaging in *Xenopus* leveraging AOD-based RAMP microscopy’s major benefit of being able to rapidly record a limited number of points in any position in a field of view. Therefore, my first goal was to generate and validate the tools, techniques and imaging platform to be able to record activity across a single, fluorescently labeled tectal neuron. Next, I wanted to apply this capability to investigate how a neuron tunes based on environmental stimuli. Rather than record the structural and functional changes across a single subsection of the dendritic arbor and then from there extrapolate those results into a broad set of principles of plasticity for the entire neuron, I wanted to be able to record and analyze all the structural and functional changes that occurred before and after a plasticity-inducing stimulus was applied. From this, I aimed to develop a set of generalizable rules to understand how learning occurs on a cellular level.
Chapter 2: Fast in vivo imaging across the dendritic arbor

2.1 Introduction

How a neuron processes information from synaptic input to its dendritic arbor and encodes the higher-order transformed information in action potential firing output is the fundamental question underlying our understanding of every mental process. There is still much that is unknown about these events in the mature brain, when neurons have well-defined receptive field tuning and circuits are largely stable. Even less is known about the mechanism by which a neuron in a developing brain establishes its dendritic arbor, selects the complement of synapses with upstream inputs, and first established its receptive field tuning properties to particular sensory inputs. Progress in this field has been stymied by the lack of technologies allowing in vivo recording of sensory-evoked neural activity across the entire dendritic arbor and soma to correlate with measures of dendritic growth and synaptogenesis. Current research involving synaptic activity sampling is limited to either in vitro experiments (H. Lee et al., 2016), or sampling discrete sections of dendritic segments in vivo (El-Boustani et al., 2018). Furthermore, what in vivo research exists is generally focused on analyzing dendritic integration in established circuits in adult animals (El-Boustani et al., 2018; Kerlin et al., 2019), leaving open the question as to how these neurons get initially wired into these circuits early in development. To that end, my goal was to elucidate how neurons in an awake, developing brain employ sensory experience to alter growth and synaptogenesis in order to develop or refine tuning properties. Using the visual system of the albino Xenopus laevis tadpole as an accessible vertebrate model of early brain circuit formation, I aimed to understand how sensory-driven neural activity directs dendritic arbor remodeling to improve encoding properties.
2.2 Designing a customized calcium sensor

To image synaptic and action potential activity in individual tadpole tectal neurons it is necessary to utilize an activity sensor capable of sampling both synaptic input and action potential output. To accomplish this, I utilized the latest generation of Genetically-Encoded Calcium Indicators (GECIs), jGCaMP7s, which compared to other GECIs, boasts an unparalleled sensitivity and dynamic range (Dana et al., 2019). In order to improve its sensitivity to detecting discrete localized synaptic events, I modified jGCaMP7s to incorporate a short sequence to the C-terminus for post-translational farnesylation, which promotes targeting to the plasma membrane. Farnesylation has been previously demonstrated in *xenopus* to effectively target fluorophores to the cell membrane (S. X. Chen et al., 2012). However, one drawback to utilizing jGCaMP7s is that its resting-state fluorescence is relatively dim compared to EGFP, precluding its utility for imaging complete dendritic arbor structures. Thus, it was necessary to develop a bright, space-filling, bleach resistant fluorophore that could be co-excited with jGCaMP7s, but with a different emission spectrum to prevent signal mixing. This fluorophore would act as a “space-filler” that enable the identification of small dendritic structures such as filopodia, where previous research has show there is an especially high concentration of synapses (Li et al., 2011; Ruthazer & Aizenman, 2010; Sin et al., 2002).

The ideal space-filling fluorophore was a red light emitting fluorophore as this would allow for easy separation of emitted light from jGCaMP7s with off-the-shelf emission filters, and there is a wide array of red fluorophores developed and publicly available. To be able to be successfully utilized as a space-filler for single-neuron imaging over multiple time points, in addition to being bright, the fluorophore must also be photostable. I found that the red fluorophore mCyRFP1 was
optimal since, in addition to being bright and stable, has the advantage of having an exceptionally large Stokes shift, which had previously been employed by utilizing mCyRFP1 as a Fluorescence Resonance Energy Transfer (FRET) partner capable of being excited simultaneously with EGFP based biosensors (Laviv et al., 2016). This allowed for efficient excitation near 900nm, which is within the NIR optical window for minimal light absorption and scattering in tissue (Weissleder & Ntziachristos, 2003), while also efficient for the excitation of jGCaMP7s (Laviv et al., 2016). mCyRFP1 was also designed to be monomeric which was important to prevent aggregation of the fluorophore and potentially form subcellular vacuolar inclusions when expressed in Xenopus tectal neurons. I next created a membrane-localized variant of mCyRFP1 by addition of a C-terminal farnesylation sequence to further enhance mCyRFP1’s ability to brightly label neurons to achieve complete images of small dendritic processes.

Now that I had both an optimized green activity sensor in farneslyated jGCaMP7s and space-filling red fluorophore in farneslyated mCyRFP, the challenge was to ensure that both were expressed in a single neuron at levels high enough to be useable for imaging experiments. In order to assure that both the green calcium indicator and red space-filler fluorophores are co-expressed, I built a plasmid in which expression of both farneslayted jGCaMP7s and farneslayted mCyRFP are driven by the same promoter, but the fluorophores are separated by a P2A sequence (Ahier & Jarriault, 2014; Z. Liu et al., 2017). This sequence encodes a short peptide that self-cleaves after translation. This system ensures dual expression of two independent proteins at an exact 1:1 ratio and it has the added benefit that verification of the expression of one fluorophore consequentially ensures expression of the other fluorophore on the P2A expressing plasmid. This is extremely useful since the baseline fluorescence of jGCaMP7s is quite dim. Thus, through utilizing my jGCaMP7s-P2A-mCyRFP1 plasmid, I am able to ensure that both fluorophores will be adequately
expressed when electroporated into a neuron while also ensuring that they can act independently of each other.

At the time, the P2A construct had been tested to be functional in cells derived from a variety of model organisms, including, mice, *c. elegans* and zebrafish, however it had not been validated in *Xenopus* tadpoles (Ahier & Jarriault, 2014; Kim et al., 2011; Z. Liu et al., 2017). To validate the proper cleaving of P2A in *Xenopus* I designed a plasmid where a green fluorophore, sfGFP had a c-terminus nuclear exclusion signal (NES) added and was placed upstream of the P2A sequence and a red fluorophore, TagRFPT was localized subsequent to the P2A and had a nuclear localization signal (NLS) added. The entire expression cassette was under the control of a strong promotor, pCAG to ensure strong expression of the fluorophores. Using whole-brain electroporation, I expressed this construct in multiple cells throughout the tadpole tectum and then imaged the expression after 48 hours (Fig. 2-1A). I observed that the red signal was indeed localizing to the nucleus and the green signal was nuclear excluded, which established that the P2A peptide was indeed functioning, as both fluorophores were able to localize independently. To confirm the proper function of the P2A, I performed a western blot where I expressed either a version of this strong-expression dual-colour plasmid that lacked the nuclear or an untransfected control in the brains of 15 tadpoles per condition (Fig. 2-1B). When I blotted for GFP, I found that the band produced in the construct of the strong promotor was located at the size that corresponds to the size of the cleaved version of the protein (29kd) as opposed to the size of the uncleaved version (56kd). Thus, I had strong evidence from both biochemical and functional tests that the P2A self-cleaving peptide functioned properly in *Xenopus* tadpoles.
Figure 2-1

Testing the function of a P2A self-cleaving peptide in *Xenopus* tadpoles. (A-Aii) Tadpoles expressing a pCAG-nes-sfGFP-P2A-TagRPPT-NLS were imaged 48 hours post whole brain electroporation. (B) A western blot was performed using anti-gfp against the brain tissue of tadpoles that expressed either expressing a pCAG -sfGFP-P2A-mCyRFP or untransfected controls.

2.3 Methods
2.3.1 Plasmid design

A C-terminal nuclear localization exclusion (Dang & Lee, 1988) and an N-terminal nuclear localization sequence (Ding et al., 2015) was added to a plasmid expressing sfGFP-P2A-mCyRFP (From the Brian Chen Laboratory) using PCR based restriction-free cloning. N-terminal farnesylation sequences (derived from Clonetech product #6074-1) were added to jGCAMP7s (Addgene # 104463) and mCyRFP (Addgene # 84545) using PCR based restriction-free cloning. Using Gibson Assembly (New England Biolabs product # E5510S) the farnesylated jGCAMP7s, farnesylated mCyRFP and a P2A sequence between the two fluorophores was added to a plasmid backbone under with the pCAG promotor (derived from Addgene # 13775).

Nuclear localization sequence:

GCTCCCGATATCAAGACCTGCTAATTTCAAGGCTAA

Nuclear exclusion sequence:

GTCTAGTTTAACGCGTTTGGCAGCAGG

Farnesylation sequence:

AAGCTGAACCCTCCTGATGAGAGTGGCCCCGGCTGCATGAGCTGCAAGTGTG

TGCTCTCCTGA

2.3.2 Expression of plasmid constructs

To express plasmid constructs across a large number of neurons, whole-brain electroporation was employed (Haas et al., 2002). Whole-brain electroporation parameters for
plasmid expression were five square pulses with a duration of 50 ms at 20V delivered at a rate of 1 pulse per second.

2.3.3 Protean extraction and western blotting

The optic tecta of fifteen tadpoles expressing pCAG -sfGFP-P2A-mCyRFP and fifteen untransfected controls were dissected, lysed with 1X RIPA buffer and mechanical processing and spun down using a centrifuge and the protein was quantified. 100μg of protein was loaded onto a gel for SDS-PAGE and subsequently transferred onto nitrocellulose. The blot was probed with anti-GFP (R&D systems product # AF4240).

2.3.4 In vivo imaging

Stage 48/49 Albino *Xenopus laevis* tadpoles expressing the plasmid construct were bathed for 5 minutes in 4mM pancuronium to temporarily paralyze them immediately prior to imaging. The tadpoles were then placed in an imaging chamber that was perfused with oxygenated 0.1x Steinberg’s solution for the duration of the experiment. Imaging was performed using a galvometric mirror based two-photon scanning microscope with a tuneable laser (Coherent Chameleon Vision II) emitting at 910nm, and two output PMTs (Hamamatsu H7422-40) with red (Chroma ET 590/50) and green (Chroma 525/50) emission filters.
Chapter 3: Manuscript 1- Comprehensive imaging of sensory-evoked activity of entire neurons within the awake developing brain using ultrafast AOD-based random-access two-photon microscopy

3.1 Preface: Fast sampling of neural activity in an *in vivo* model

Now that I had the genetic tools for being able to visualize whole-neuron activity while also brightly labeling fine structures in the dendritic arbor, the next challenge was to develop a platform capable of leveraging these tools. There was a need to develop an imaging system capable of sampling neuron activity at rates sufficient to detect synaptic and somatic calcium events as well as recording structural changes in the dendritic arbor. To investigate how sensory experience directs neuronal growth, synaptogenesis and receptive field development, a major challenge resides in efficiently scanning and analyzing the activity of the complete arbor of individual neurons *in vivo*. While high-resolution analysis of calcium activity in cultured neurons is relatively straightforward using fast wide-field or confocal imaging (H.-K. Lee et al., 2000), attempting to perform similar experiments of complex 3D neural structures in conscious vertebrates required volume scanning at velocities sufficient to track changes in calcium dynamics, on the order of tens to hundreds of milliseconds. Conventional laser-scanning two-photon microscopes relying on galvo-mirrors for positioning excitation laser focus in the 2D x/y-axes image plane, and motors for stepping through the z-axis are too slow for comprehensively imaging even relatively small tectal neurons whose dendritic arbors span 100 μm x 100 μm x 100 μm.

Fortunately, several people in the Haas laboratory had been working to solve this problem. Drs. Kaspar Podgorski and Kelly Sakaki had been developing an *in vivo* fast imaging platform that utilized “Random Access Multi-Photon (RAMP) Microscopy” (Bullen et al., 1997; Katona et al., 2012) to reduce the number of points sampled during scanning to only those that contained the
neuron, thus greatly increasing the sampling rate. I aided in the construction of the final version of this imaging system that was designed by Dr. Kelly Sakaki and then performed the experiments to validate and benchmark the performance of the platform. This platform was capable of sampling a whole neuron several times per second or individual planes at hundreds of times per second. With the aid of a fellow graduate student, Patrick Coleman, we designed a Segmented Scanning strategy in which a dendritic arbor is divided into 3 compartments. Each third is serially imaged allowing rates of 6-10Hz and then the total neuron is stitched back together. It was this Segmented Scanning strategy that would become the key tool in subsequent experiments investigating neural plasticity across the complete dendritic arbor.
3.2 Summary

Determining how neurons transform synaptic input and encode information in action potential (AP) firing output is required for understanding dendritic integration, neural transforms and encoding. Limitations in the speed of imaging 3D volumes of brain encompassing complex dendritic arbors in vivo using conventional galvanometer mirror-based laser-scanning microscopy has hampered fully capturing fluorescent sensors of activity throughout an individual neuron’s entire complement of synaptic inputs and somatic APs. To address this problem, we have developed a two-photon microscope that achieves high-speed scanning by employing inertia-free acousto-optic deflectors (AODs) for laser beam positioning, enabling random-access sampling of hundreds to thousands of points-of-interest restricted to a predetermined neuronal structure, avoiding wasted scanning of surrounding extracellular tissue. This system is capable of comprehensive imaging of the activity of single neurons within the intact and awake vertebrate brain. Here, we demonstrate imaging of tectal neurons within the brains of albino Xenopus laevis tadpoles labeled using single-cell electroporation for expression of a red space-filling fluorophore to determine dendritic arbor morphology, and either the calcium sensor jGCaMP7s or the glutamate sensor iGluSnFR as indicators of neural activity. Using discrete, point-of-interest scanning we achieve sampling rates of 3 Hz for saturation sampling of entire arbors at 2 μm resolution, 6 Hz for sequentially sampling 3 volumes encompassing the dendritic arbor and soma, and 200-250 Hz for scanning individual planes through the dendritic arbor. This system allows investigations of sensory-evoked information input-output relationships of neurons within the intact and awake brain.
3.3 Introduction

Neurons within brain circuits receive inputs from hundreds to thousands of upstream neurons at synaptic contacts distributed across their elaborate dendritic arbors. Information received in synaptic currents is integrated within dendrites and transformed into neural output encoded in action potential (AP) firing at the soma, which is then transmitted via the axon to downstream targets. Information carried by neural activity can be deciphered by tracking synaptic events or APs evoked by controlled sensory stimuli in awake animals. However, our understanding of neural information processing and encoding is limited due to technical challenges in simultaneously tracking activity throughout complex three dimensional (3D) dendritic arbor structures and the soma, which is needed to establish full input-output relationships.

Approaches to this problem have been aided by the development of fluorescent sensors of neural activity, particularly those capable of detecting rapid changes in intracellular free calcium. Neurons tightly control the transmembrane calcium gradient with intracellular concentrations being on the order of 10,000 times lower than extracellular levels (Gleichmann & Mattson, 2011). Synaptic and AP activity evoke distinct spatiotemporal elevations in intracellular calcium (reviewed in (Redmond & Ghosh, 2005; Sabatini et al., 2002)). Excitatory, glutamatergic synaptic transmission induces calcium influx by activation of calcium-permeable N-Methyl-D-aspartate (NMDA) and GluA2 subunit-lacking α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtypes of glutamate receptors (Burnashev et al., 1992), and secondary opening of voltage-gated calcium channels (Cowan et al., 2001; Oertner & Svoboda, 2002; Sabatini et al., 2002; Sabatini & Svoboda, 2000). Transients within the soma from voltage-gated calcium channels are also triggered by AP-mediated depolarization (T. Chen et al., 2013; Gleichmann & Mattson, 2011; Nakai et al., 2001). Thus, calcium transients can be imaged as a proxy for neural
activity using calcium-sensitive fluorescent dyes or genetically-encoded calcium indicators (GECIs) (Nakai et al., 2001; Rose et al., 2014), such as the family of engineered GCaMPs (T. Chen et al., 2013). These indicators inherently slow neural activity signals due to their calcium binding kinetics (T. Chen et al., 2013; Nakai et al., 2001), but require sampling rates on the order of milliseconds to adequately measure transient rise times and signal intensity (Katona et al., 2012), and seconds for decay times (T. Chen et al., 2013; Sun et al., 2013). An alternative strategy for detecting synaptic input has been the development of sensors of neurotransmitters. For sensing glutamate, the genetically encoded protein iGluSnFR can be expressed on the surface of neurons and increases fluorescence upon binding to presynaptically-released glutamate (Marvin et al., 2013, 2018).

In order to fully capture a neuron’s synaptic and AP neural activity within the intact brain one must image the complex 3D dendritic arbor morphology and soma relatively deep within light-scattering brain tissue at sample rates sufficiently fast to track activity. Conventional two-photon laser scanning microscopy (TPLSM) systems achieve deep imaging up to 600-800 µm into brain through use of long-wavelength excitation light which can penetrate further into the brain than wavelengths used for single-photon absorption in confocal microscopy (Denk et al., 1994; Svoboda et al., 1997). However, the most significant obstacles of conventional TPLSM systems in achieving full imaging of brain neuronal activity is their slow rate of 3D-volume imaging. Inertia-induced latencies are incurred by the galvanometer mirrors used to deflect the laser between imaging points. Conventional systems image brain neurons (e.g. within volumes of 100 µm³) using numerous sequences of line-scans (i.e. ‘rastering’) to image a single 2D X-Y image-plane. Repeating this 2D sampling at multiple incremental steps throughout the Z-axis is employed to fully capture the 3D-volume encompassing the neuron’s cell body and dendritic arbor. Although
conducting line-scans using galvanometer mirror-based systems is relatively fast between two points of interest (POIs) on one X-Y plane, requiring only single-axis transitions, scanning rates slow dramatically as the number of nonlinear POIs increase due to multiple X-, and Y-axis transitions. For example, imaging two points on a 10-µm line on the same plane can achieve scanning rates of 100 Hz attainable reduces to 20 Hz, yet decreases to 1 Hz or slower when the points are spread over an area of 100 µm². Thus, imaging a 3D volume of 100 µm³, with 1µm interval steps per plane on the Z-axis (i.e. 100 X-Y image planes) requires in excess of 100 seconds per cycle to complete, which is significantly below the rate required for tracking neural activity using calcium biosensors. Critically, the vast majority of sample time conducted by such conventional TPLSM volume imaging of brain neurons is wasted since the structure of neurons comprises a small fraction of the image space. Since neuronal morphology is comprised of long, thin and often highly branched dendrites, the majority of the 3D volume is composed of irrelevant unlabeled extracellular tissue surrounding the target neuron.

Significant recent advances in microscopy have improved the spatial and temporal sampling rates of conventional imaging systems, which have been critical steps towards achieving comprehensive imaging of brain neurons. To increase the ability to change focal depths piezo-actuators have been employed, such as driving the actuator with a sinusoidal voltage, to produce rapid mechanical oscillation of the microscope objective (Göbel et al., 2007). Combined with galvanometer mirror control of the focal point in the X-Y plane, such systems proved capable of capturing somatic calcium transients from a volume containing several hundred cell neurons at 10 Hz. Incremental increases in imaging speeds can be achieved by replacing X-Y rastering with a faster spiral scan pattern, or restricting imaging areas to pre-defined areas containing neuronal elements. Further improvements were achieved by synchronizing the X-Y positioning by the
galvanometer mirror with the Z-axis oscillatory motion of a piezo-actuator to scan 3D neuron structures (Katona et al., 2011).

Significantly increased rates of imaging complex 3D neuronal structures have been achieved using ‘random-access’ sampling, which allows discrete Point of Interest (POI) sampling, and scanning multiple POIs in 2- or 3D without scanning the intervening space between them (Fig. 3-1). Random access microscopy takes advantage of acousto-optic deflectors (AODs) in place of galvanometer mirrors (Bullen & Saggau, 1997, 1999; Grewe et al., 2010; Iyer et al., 2003; Katona et al., 2012; Nadella et al., 2016; Reddy et al., 2008; Salomé et al., 2006; Szalay et al., 2016). Soundwave-mediated changes in the AOD crystal refractive index acts as a diffraction grating to refract the path of the laser, producing a rapid, inertia-free laser position transition system. Systems using pairs of AODs can achieve kHz-rate sampling in X-Y planes for both one- and two-photon imaging (Bullen & Saggau, 1997, 1999; Iyer et al., 2003; Salomé et al., 2006), and two pairs of AODs can be used for X-Y and Z-axes transitions (Katona et al., 2012; Nadella et al., 2016; Reddy et al., 2008; Szalay et al., 2016). AODs have also been recently applied as laser beam shapers, which could serve systems that require very fast wavefront control (Akemann et al., 2015). AOD-based microscopy has been applied to imaging multiple dendrites and the soma of individual neurons in vivo in rodents (Szalay et al., 2016). Fast imaging of cubes or rectangular volumes encompassing the target neuronal compartments was used to compensate for the motion artifacts inherent in mammalian models due to blood flow and respiration. Other approaches incorporate remote focusing to speed Z-axis positioning enabling line-scanning in vivo within a volume spanning over two hundred microns (Nadella et al., 2016).
Figure 3-1

Comprehensive imaging. (A) The random-access, two-photon laser-scanning system used to analyze neuron morphology and functional activity. (B) Schematic of *Xenopus* tadpole brain visual circuit showing tectal neurons targets (green) for comprehensive imaging *in vivo*. Neurons are labeled with a space-filling fluorophore to capture morphology and a calcium indicator to monitor activity. Neuronal morphology is first determined using a stack of 2D planes.
encompassing the entire neuron. (C) Next, the user traces the 3D dendritic arbor of the neuron, from which points-of-interest (POI) along the entire dendritic arbor and cell body are converted to a 3D tree-structure relationship, and (D) automatically interpolated at 2 μm intervals. A “rapid-scan” executes a routine employing AOD-based random-access imaging to simultaneously sample the interpolated POIs to record the AP and all synaptic activity across the neuron.

However, application of fast-scanning technologies for comprehensive imaging of fluorescent indicators of sensory-evoked neural activity across an entire neuron in vivo remains a challenge. Here, we approached this problem by designing a random-access AOD-based TPLSM system with a variable-speed piezo-actuator system for Z-axis focus, combined with a priori determination of the neuronal structure to direct the image path, as well as by selecting a model system with minimal drift and brain neurons with relatively small dendritic arbors. We have selected the albino Xenopus laevis tadpole as a model system since they are readily immobilized under awake conditions, and their transparency allows direct visualization of neurons without the brain tissue movement intrinsic to mammals. We fluorescently label tadpole brain neurons using in vivo single-cell electroporation to transfect neurons with space-filling fluorophores or biosensors of activity (Haas et al., 2001). Our approach involves first imaging the brain volume encompassing a target neuron using serial sectioning (Fig. 3-1C), and then creating a schematic rending of the soma and dendritic arbor to provide the a priori knowledge of the full 3D neuronal structure to direct discrete fast random-access sampling (Fig.3-1D). This a priori structure must be determined for each neuron imaged, since individual brain neurons have unique patterns of dendritic branches extending in non-uniform paths (S. X. Chen et al., 2010; X. Chen et al., 2011; Haas et al., 2006; Hossain et al., 2012; Romand et al., 2011). We provide examples for application
of our AOD-based random access TPLSM system for imaging of visual-evoked neural activity of individual brain neurons within the optic tectum of awake, immobilized tadpoles. We describe the software driving this microscope and alternative modes of sampling neural activity to optimize coverage or sample rate.

3.4 Methods

3.4.1 AOD-based random-access microscope optical train

The optical train (Fig. 3-2) of the random-access system achieves high-throughput POI scanning as well as volume imaging (i.e. multiple X-Y images within a volume region) by using a piezo-actuator, for Z-axis motion, and two AODs to provide X-, Y- axis scanning. In combination, this allows for sampling at any possible scan points in three dimensions. In our system, the X and Y axes were scanned using two wide-scan angle AODs, $AOD_{1,2}$ (OAD1121-XY deflector / DA104-2 power driver, Isomet) for NIR with a scan angle of 5.4 degrees, an $T_{ACC}$ of 13 $\mu$s, 40 MHz bandwidth and 9 mm apertures. Transitioning between any two points on the image plane using the AODs executes at rates up to 100 kHz. A Ti:Sapphire laser (Chameleon Vision II, Coherent), with tuned, temporal-dispersion compensation, provides femtosecond pulses and enters the preprocessing optical train through a dichroic mirror $D_1$ (T660LPXR-UF2, Chroma) and overfilling the back-aperture of a water immersion objective (60X, 1.1 NA, 0.150 kg, LUMPL, Olympus). The objective is mounted on a piezo-actuator ($PA_1$, QNP-250-250L, 250 $\mu$m range, 1 kg maximum payload, Aerotech Inc.) to adjust the focal plane.
Figure 3-2

Optical train and the stimulation module. (A) The pre-processing optical train for the AOD TPLSM undergoes 4X beam expansion and is passed through a prism used to spatially compensate for the dispersion created in the acousto-optic deflectors deflecting the beam into the back aperture of a 60X objective. (B) The beam enters the optical train of the AOD-TPLSM, passes through (C) two AODs orientated 90 degrees with respect to each other and provides X-Y scanning. Two lenses collimate the laser at the pupil of the back aperture of the microscope objective. The fluorescence light returns through the objective and (D) enters the post-processing optics by reflecting off a dichroic mirror. The emission path on the AOD-TPLSM is split between two
channels using a short-pass dichroic mirror. Channel 1, filtered green emissions, and Channel 2 captures red emissions. Calcium transients were observed using jGCaMP7s on the Channel 1. The fluorescence light is detected by a PMT dedicated to each channel.

The fluorescent light emitted from the sample returns through the objective and exits from the back aperture pupil, reflects off $D_1$ and is then filtered through a shortpass filter ($SPF_1$, 700SP-2P, Chroma) to prevent stray laser emissions. The emitted and collimated light is focused using the relay lens $L_5$, and then separated into green and red wavelengths by a dichroic mirror, $D_2$ (565LPXR, Chroma). The green channel, to measure the jGCaMP7s signal, is filtered using a bandpass emission filter ($EF_1$, ET525/50m-2P, Chroma) and red channel is filtered using a bandpass emission filter ($EF_2$, ET620/60m-2P (Chroma). The emitted light from each channel passes through the relay lenses, $L_6$ and $L_7,8$, to fit through the apertures of the GaAsP photomultiplier tubes (H7422-40, Hamamatsu). The PMT signals are amplified using low-noise current amplifiers (SR570, Stanford Research Systems). The signal output of the amplifier was acquired using a 12-bit analog input (PCI-6110 DAQ; National Instruments), at a rate of 2.5 MHz.

3.4.2 Piezo-actuator and POI-based trajectory

The primary role of the Z-axis actuator for comprehensive imaging is to move the focal plane of the objective to each POI between [$Z_{\text{min}}$, $Z_{\text{max}}$] on the interpolated neuron in 3D space (Fig. 3-3A). Piezo-actuators are capable of large accelerations with heavy loads over relatively large distances and can provide sub-micrometer repeatability. For these reasons, a piezo-actuator was used to provide stable position-control for planned, POI trajectories during the RS. In contrast with certain previous fast-scanning approaches in which a piezo-actuator was used to adjust the focal
plane through employing a sinusoidal vibration of the objective for the purposes of sampling a populations of multiple neurons (Göbel et al., 2007), we chose to implement a unique single ‘sweep’ trajectory for each neuron. This method was chosen because of the non-uniform distribution of POIs on the Z-axis due to unique and sporadic patterns of dendrite branching of each brain neuron’s dendritic arbor. Our routine calculates the required trajectory to scan all POIs using a monotonically increasing path from \(Z_{\text{min}}\) to \(Z_{\text{max}}\) (Z-axis distance encapsulating all of the POIs) varying the position of the piezo-actuator to adjust the focal plane. The duration of the trajectory is based on the mechanical limitations (maximum velocity and acceleration) of the piezo-actuator and the required time the focal plane should exist at any particular position on the Z-axis to scan one or more POIs.

Figure 3-3
POI distribution and Z-actuator schedule and trajectory. (A) A typical distribution of POIs in a tectal neuron and the scanning schedule of POIs synchronizing the activity of the Z-actuator and the AODs. (B) An illustration of the neuron structure representing the POIs scanned in A, and an example of three generic POIs on the neuron in 3D space and how they relate to a possible Z-
stage trajectory planned for scanning and synchronization with the AODs (inset). The “short” line scan of each POI is created in image space using 11 pixels. The center pixel exists at the location of the POI and each pixel is created from 5 summed samples of the PMT output.

### 3.4.3 AODs and high speed POI scanning

AODs are ‘solid-state’ devices that can provide random-access control of laser beam positioning at high speeds and have negligible fly-back delay in comparison to scan mirrors. The ‘laser-scanning’ mechanism of an AOD consists of passing a soundwave through a piezoelectric transducer coupled to a crystal (e.g. TeO$_2$). Controlled changes in the piezoelectric modulate the crystal causing contractions and rarefactions in the substrate resulting in periodic changes in the refractive index similar to a diffraction grating, to refract the path of the laser. The deflection angle of the AODs, $\theta_{\text{scan}}$, can be modulated using the following relation,

$$\theta_{\text{scan}} = \frac{\lambda f_{\text{mod}}}{v}$$

where $\lambda$ is the wavelength of the laser, and $f_{\text{mod}}$, is the modulating frequency passing through the AOD crystal and $v$ is the intrinsic, acoustic velocity of the material. The rate of change of the AOD is limited to the ‘access time’, $T_{\text{ACC}}$, the time for the radio-frequency wave to propagate completely through the beam waist of the laser in the AOD deflecting the path of the laser to a different angle. $T_{\text{ACC}}$ is defined as,

$$T_{\text{ACC}} = \frac{\phi_L}{v}$$

where $\phi_L$ is the laser beam diameter.
3.4.4 POI Scan

Each POI scan (Fig. 3-3B) consists of a short line scan of 11 pixels, and 5 samples per pixel. The 5 measurement samples per pixel site are summed, and the combined intensity $I(k)$ is then mapped to image space $I(x,y)$. This is done to account for the spatial drift and noise that is inherent in in vivo imaging experiments. The minimum time required to scan a POI is defined by the following sum,

$$T_{POI} = T_{Buffer} + T_{ACC} + T_{SCAN}$$

where $T_{BUFFER}$ is the time required between samples and $T_{SCAN}$ is the time for the AODs to scan from $f_0$ to $f_1$ shifting the path of the laser. The maximum allowable velocity of the piezo actuator, $V_{Z,MAX}$, is constrained by $T_{POI}$ where a required tolerance, $d_{TOL}$, is defined to ensure the laser excites the coordinates of the POI to capture the fluorescent signal. $V_{Z,MAX}$ is defined as follows,

$$V_{Z,MAX} = \frac{2d_{TOL}}{T_{POI}}$$

3.4.5 AOD POI laser deflection programming

The relationship between image space coordinates and the AOD frequency input converts image POI coordinates to AOD instructions, which control the random-access, laser excitation position on the focal plane. Image space coordinates (i.e., locations of the POIs) are converted to AOD frequencies using the following,

$$\begin{bmatrix} f_0 \\ f_1 \end{bmatrix} = \begin{bmatrix} f_{max} - f_{min} \\ f_{BW} \end{bmatrix} \begin{bmatrix} u_0 - \frac{L_{POI}}{2} - 0.5 \\ u_1 + \frac{L_{POI}}{2} + 0.5 \end{bmatrix} + [f_{min}]$$
where each pair of frequency instructions \( (f_0, f_1) \), commands the AODs to execute a swept range from \( f_0 \) to \( f_1 \), between the minimum frequency of the AOD, \( f_{\text{min}} \), to the maximum frequency, \( f_{\text{max}} \). The swept range represents a short line scan and directs the laser through the POI on the arbor of the neuron to excite fluorophores and capture the resulting fluorescence light sample, \( I(k) \). \( u_0 \) is the start of the line scan and \( u_1 \) is the end of the line scan in image space. \( L_{\text{POI}} \) is the length of the line scan, 11 pixels in image space. Since AODs are mounted orthogonally with respect to each other, duplicate coordinate pairs are sent to each other resulting in a diagonal sweep across the imaged points \( u_0, u_1 \), in the X-Y plane.

3.4.6 Piezo-actuator and AOD Synchronization

The position of the piezo-actuator and deflection angle of the AOD are synchronized to coordinate the laser focal point on the neuron’s dendritic arbor and cell body in 3D space to excite the fluorophores and detect the fluorescence emissions (Fig. 3-4). This is achieved by acquiring a template sweep trajectory scan (prior to executing the RS) and scheduling each frequency pair instruction (i.e. X-Y-position of the laser on the focal plane) according to the time of the known position of the piezo-actuator (i.e., Z-position of the focal plane). The template scan is generated by executing the planned trajectory repeatedly over a 4 second interval providing a recorded output of the piezo-actuator position feedback. Using the position feedback, an AOD start-pulse signal schedule is generated for each frequency pair. Each start-pulse synchronizes the start of the frequency pair instruction in the AODs with the position of the piezo-actuator to ensure each 11-pixel line scan crosses through each POI.
Figure 3-4

Timing diagram for system synchronization. Synchronization between devices is coordinated using the sample clock signal, $T_{CLK}$. Initially, the start signal ($START$) is activated indicating the beginning of the measurement sequence. All PMT channels initiate recording and AOD “chirps,” which are responsible for deflecting the path of the laser for line scans for F3D or FS measurement sequences. The Z-stage command signal, $CMD-Z$, begins after $START$, and feedback
is recorded providing an absolute comparison between the desired position and actual position measured.

3.4.7 System requirements

We experimentally determined the key temporal and spatial system requirements using image sampling of neural activity evoked by visual stimuli in the developing brain of the *Xenopus laevis* tadpole (Fig. 3-1B). Baseline measurements were conducted on a conventional galvanomirror-based TPLSM (custom-designed, modified Olympus BX61, running Olympus Fluoview 1000).

3.4.8 Temporal sampling considerations

Four temporal measurements were required in order to determine temporal scanning rates necessary to sample brain neuron synaptic and AP activity. These included:

1) $\bar{\tau}_{Delay}$; the average delay time between the beginning of the visual stimuli and the peak amplitude of the evoked intracellular calcium transient response,

2) $\bar{\tau}_R$ and $\bar{\tau}_F$; the mean time constants of the rising and falling times of the calcium transients;

3) $\bar{f}_{max}$; the frequency bandwidth of the evoked, calcium signals.

When initially designing our random-access TPLSM system, we calculated the temporal sampling considerations based on the 6th generation GCaMPs that were at the time, the latest publicly available. We experimentally obtained the temporal data of the system from neurons expressing GCaMP6m in the optic tectum of head-mounted tadpoles. Calcium transients in dendrites and the
soma were evoked by light stimuli produced by an LED. We subjected the tadpoles to repeated 50 ms OFF square pulses presented at 8-10 second intervals. Evoked responses were sampled at five locations on the neuron’s dendritic arbor and at the soma. Averaged responses from 4 OFF events were used to determine the delay between stimulus onset and event peak ($\bar{\tau}_{\text{Delay}}$) as well as the parameters for the temporal rise to peak ($\bar{\tau}_R$) and fall to the baseline ($\bar{\tau}_F$). $\bar{\tau}_{\text{Delay}}$, $\bar{\tau}_R$ and $\bar{\tau}_F$ were recorded experimentally as 0.404 s, 0.237 s and 2.907 s respectively. (Fig. 3-5A).
Figure 3-5

Visual-evoked calcium responses in a brain neuron using GCaMP6m. (A) A line scan of the neuron's soma was acquired at 1,000 Hz showing the evoked response following a 50 ms light
The maximum intensity peak was 0.404 s after the OFF pulse. The rise time parameter (at intensity 1-1/e of peak) was measured 0.237 s after the OFF pulse, and the decay time parameter (intensity 1/e of peak) occurred 2.907 s after the peak of the signal rising transient. **(B)** Frequency response of **(A)** after applying the Fourier transform. Most information is in the very low frequency range (under 2 Hz), with diminishing power until 7 Hz, and noise thereafter.

A Fast Fourier Transform (FFT) was then applied to each time-domain signal to acquire the frequency domain response. The average of all FFTs (Fig.3-5B) indicated most of the signals existed around 3 Hz. Thus the Nyquist sampling rate, $f_{Nyq}$, during comprehensive imaging should be at least 6 Hz. Subsequent to the release of the 7th generation of GCaMPs we switched to using jGCaMP7s, which has both superior sensitivity and a slower decay rate than the 6th generation GCaMPs (dal Maschio et al., 2012; Dana et al., 2019). This allows for a potentially slower Nyquist sampling rate than previously calculated. When advanced spike deconvolution methods are required to achieve millisecond-precision on spike timings (Deneux et al., 2016), the faster planar scans are recommended due to the 200 Hz time resolution, and faster indicators such as jGCaMP7f can be used.

### 3.4.9 Spatial sampling considerations

The requirement to capture POIs distributed across an entire neuron within the intact and awake brain requires imaging a large enough field-of-view to encompass the entire 3D dendritic arbor and the cell body over long periods. The minimum POI spatial resolution ($d_L$) along the
dendritic arbor is experiment-dependent and limited by scanning speed; however, should be less than 5 µm to identify neuronal sub compartments (Biess et al., 2011) and to saturate the domains of calcium transients allowing us to discriminate individual events. Thus, the optical resolution is required to be less than 2.5 µm to satisfy the Nyquist spatial sampling requirement.

3.4.10 Temporal and spatial scanning limitations

The temporal and spatial scanning limitations define the rates for the minimum scanning frequency, \( f_{Nyq} \), required for calcium imaging. This rate will vary based on the number of POIs, \( N_{POI} \), on each unique neuron arbor and is a function of the total dendritic branch length, \( L_T \) and the spatial resolution, \( d_L \). The minimum POI scanning frequency, \( f_s \), required can be calculated as follows,

\[
f_s \geq 2 f_{Nyq}
\]

\[
f_{Nyq} = \left( N_{POI} \cdot T_{POI,SCAN} \right)^{-1} \approx \left( \frac{L_T}{d_L} \cdot T_{POI,SCAN} \right)^{-1}
\]

\[
f_s \geq 2 \left( \frac{L_T}{d_L} \cdot T_{POI,SCAN} \right)^{-1}
\]

where \( T_{POI,SCAN} \) is the time required for the system to scan one POI.

3.4.11 Microscope driver software design

One of the overarching goals for our random-access TPLSM system design was to create a modular and flexible architecture. We believed that by partitioning the system’s responsibilities and by maintaining a loosely coupled design, we would greatly simplify and expedite system
modifications in response to future needs or technological innovations. For this reason, the system was designed using the Actor Framework (AF) concept (Hewitt et al., 1973), which supports implementing large, queued-message handler systems. The system was implemented using object-oriented design/programming for the AF (e.g., National Instruments LabVIEW (Elijah, K, 2012), and allows for scalability and easier maintenance as the system expands over time. In general, the AF subdivides the system routines into ‘actors’ or primitives that respond to messages from other actors and execute local routines with a minimal amount of overhead. By keeping routines fast, this avoids long synchronous processing, which enables asynchronous event-based processing that can require precise timings.

3.4.12 Graphical User Interfaces

Interactions between the operator and the system are facilitated through a measurement user interface (i.e. ‘Msmt. UI’, Fig. 3-6A), and the Cell Trace and Analysis Kit user interface (i.e. ‘CTAK UI’, Fig. 3-6B) to define experiments (i.e. individual or sequential sets of measurements) and draw the 3D neuron morphologies for experiments. These interfaces provide access to hardware parameters, and experiment design/post-processing tools respectively.
Figure 3-6

System user interfaces. (A) The measurement user interface (i.e., Msmt. UI) provides user access for hardware selection, and experimental design. (B) The cell trace and analysis toolkit user interface (CTAK UI) serves as an interface for drawing the initial 3D structure of the neuron, correcting minor positional errors following scans, and for post-analysis following either the F3DS or FSS routines.

The Msmt. UI allows the operator to design scanning experiments, and set parameters required by the hardware peripherals. Experimental measurements (e.g., type of scan and number of repetitions of each type of scan) are arranged in order of execution, defined by the operator, and stored as an instance of the ‘Experiment’ class. Each Msmt. in the Experiment executes the following sequence of overridden methods:

1. Initialize: preliminary settings for the measurement
2. Configure: requests resources to execute the measurement
3. Acquire: records the data from the input channel(s)
4. Measure: performs calculations using the raw data
5. **Close**: Releases resources held during the acquisition stage and signals completion.

The *CTAK UI* acquires operator input during experiments and provides a visual display of the results of each *Msmt*. During experiments, the operator can draw/modify the spatial organization of the POIs defining the neuron’s dendritic arbor. This 3D structure is stored within an instance of the *Neuron* class and relates the spatial information, such as the locations of each POI, to the synaptic/AP patterns of activity recorded during comprehensive imaging (Fig. 3-1D). The *Neuron* class is a descendant of the *Tree* class and stores POIs as a conventional tree structure comprised of a doubly linked list of *Branch Node* objects.

### 3.4.13 Proposed event sequence for comprehensive imaging

Our system separates comprehensive imaging of brain neurons into a sequence of pre-defined *Msmts*. executed by the system actors. These measurements are designed to be composable, extendable steps, and those available with the system are illustrated in Fig 3-7. An initialization routine begins by executing a focus-scan (*FS*, Fig. 3-7A), a repeating X-Y scan allowing the operator to adjust the X, Y or Z-position of the specimen and set the imaging volume-boundaries encapsulating the neuron. These boundaries define the maximum limits to all scanning routines acquired during the experiment. A full 3D scan (*F3DS*, Fig. 3. 7B) is acquired and consists of a stack of *N_{IMG}* images with a resolution of *S_x*×*S_y* pixels and separation distance, *Z_{INT}*). This first *F3DS* provides the operator a representation of the neuron’s 3D arbor on X-Y image planes. In Fig. 3-7C, an illustration of one X-Y image in the stack shows the acquired cross-section of the neuron residing on one image plane.
Scan class hierarchy and scan types. (A) The focus scan (FS) allows the operator to adjust the position of the neuron in 3D-space, and defines the boundaries during scanning. (B) The Full 3D scan (F3DS) images the volume defined in the FS at an interval along the Z-axis, $Z_{INT}$, which is defined by the operator. (C) After the F3DS is acquired, the operator traces the neuron on each X-Y image planes forming a (D) skeletonized frame of the neuron in 3D space. (E) The registration points, drawn by the operator, are then linearly interpolated over the entire arbor using a spatial separation distance of $d_L$. (F) The POIs are scheduled for scanning and the volume rapid scan (V-RS) is executed scanning all interpolated POIs at calcium imaging rates. (G) Following the RS, the user has an opportunity to make adjustments to the position of the neuron in 3D space to account
for minor position shifts.

The stack of X-Y image planes allow the operator to manually trace the neuron’s full dendritic arbor and cell body in the CTAK UI, creating a skeletonized, 3D-frame (Fig. 3-1C, 3-7D). The initial 3D-frame defines key registration points along each branch on the neuron. The initial drawing of the neuron typically requires approximately 15 minutes. Given the delay following the conclusion of the operator tracing the neuron, a second F3DS routine is executed to provide minor adjustments to the position of the neuron on X, Y, and Z-axes to account for sample drift. Further ancillary F3DS’s are employed typically at 10-minute intervals throughout the experiment to detect and accommodate growth or position drift. Following the second F3DS, the operator-drawn, skeletonized, neuron is linearly interpolated along the dendritic arbor at an operator-selected resolution (e.g., 2 μm spacing) in 3D-scanned space defining the location of the POIs intended for scanning (Fig. 3-7E).

The rapid scan (RS, Fig. 3-7F) is the comprehensive, 3D scan used to acquire the synaptic activity and AP firing information along the arbor and cell body of the neuron at all of the POIs on the neuron. The RS is initiated concurrently with the visual stimulation routine. During the visual stimulation routine, stimuli are presented to the eye contralateral to the optic tectum containing the neuron. While the stimuli are being presented to the eye, functional activity is acquired. At the end of the stimulation sequence, a focus-and-shift routine (FSS, Fig. 3-7G) is executed to accommodate minor shifts with respect to the expected position of the cell body. A F3DS is then acquired and the operator can adjust either the location of individual POIs or the
location of the entire neuron if required. This sequential set of measurements, \((RS, FSS, \text{and } F3DS)\) and the visual stimulation routine continues as scheduled in \textit{Experiment}.

### 3.4.14 Rapid scanning scheme

To achieve comprehensive analysis of the entire neuron, the system requires a scanning routine to sample all POIs and minimize the period, \(T_G\), between POIs. Sufficiently fast sampling rates are required to detect the fast calcium transients mediated by synaptic activity throughout the expanse of the dendritic arbor and APs in the soma. Scanning routines must take into account that dendritic arbor morphologies are complex and the POIs along the arbor have a non-uniform spatial distribution along the Z-axis. Fully imaging a neuron’s entire set of POIs requires rapidly repositioning the relatively heavy microscope objective from focal plane-to-focal plane, and coordinating the AOD laser-deflection angle with the position of the focal plane in order to capture all POI in each plane.

### 3.4.15 System validation

Comprehensive imaging of sensory-evoked calcium activity in a visual stimulus processing neuron in the brain of the awake albino \textit{Xenopus laevis} tadpole was used to validate this new microscope system. This model animal was selected due to its transparency, which allows direct imaging of brain neurons in awake and immobilized specimens. Moreover, the external development of tadpoles permits imaging of early vertebrate brain developmental events that typically occur in the womb in mammals (Ruthazer & Aizenman, 2010; Sin et al., 2002). Development of the tadpole visual system has been extensively studied (Dunfield & Haas, 2009; Engert et al., 2002; Li et al., 2011; Sin et al., 2002). The experimental routine in Fig. 3-8, for
system validation, was used to identify a tectal neuron’s dendritic arbor morphology and observe the functional activity in response to a controlled, visual stimuli paradigm (Dunfield & Haas, 2009). The first epoch consists of 8 measurement cycles of the rapid-scan, \( N_{RS} \), with each cycle presenting 9 stimuli - 4 light-off pulses (OFF) on a bright background, a gradual transition shift from a bright background to a dark background and 4 light-on pulses (ON) on opposing background. A simplified variation of this routine removing the ON pulses and the gradual transition shift and instead having 9 OFF pulses on a bright background was subsequently created.

![Diagram of experiment protocol](image)

**Figure 3-8**

The experiment protocol used to validate the microscope. An initial stimulus, consisting of
alternating screens “ON” and “OFF” are displayed during the initial F3DS, after which the user draws the initial morphology in the CTAK UI. The RS executes immediately after the initial drawing of the neuron structure, and stimuli are displayed while recording the 3D, evoked activity in the dendritic arbor AP firing. In this stimulation routine, 9 stimuli are presented in sequence—4 OFF pulses on a bright background a transition shift from the bright background to the dark background and 4 ON pulses on a dark background. Each stimulus is displayed within an 8 s interval (Tp) and has a buffer time, Tb, before and after the stimulus.

3.4.16 Xenopus laevis preparation

Freely swimming albino Xenopus laevis tadpoles were reared and maintained in 10% Steinberg’s solution (Dunfield & Haas, 2009). In order to transfec brain neurons for expression of calcium sensors we used single-cell electroporation of plasmid DNA (Haas et al., 2001). Seven days post-fertilization, tadpoles were anesthetized using 0.01 % solution of MS-222 (A5040-25G, Sigma-Aldrich). A borosilicate micropipette (BF150-75-10, Sutter Inc.) pulled on a micropipette puller (P-97, Sutter Inc.) was backfilled with a solution containing 3 µg/µL plasmid DNA encoding the a green calcium-sensitive fluorophore, jGCaMP7s and a red space-filling fluorophore mCyRFP in calcium-free, ringers solution (in mM: 116 NaCl, 1.2 KCl, 2.7 NaHCO₃). The filled micropipette was then inserted into the optic tectum of the tadpole and an Axon 800A Electroporator delivered a train of voltage pulses to induce electroporative transfection. Prior to experimentation, tadpoles were paralyzed in a bath using 2 mM panacronium dibromide (0693/50, Tocris). All experimental procedures were conducted on Stage 49 tadpoles (Nieuwkoop & Faber, 1994) according to the guidelines of the CCAC and were approved by the Animal Care Committee of the University of British Columbia’s Faculty of Medicine.
3.4.17 Animal stimulation chamber with visual stimulator

A custom-designed imaging chamber (Fig. 3-9) is used to stabilize the head of the tadpole while imaging, to provide the specimen with oxygenated solution, and to provide visual stimuli to the contralateral eye. Stabilization of the animal is required during imaging routines to prevent positional drift by minimizing tadpole movement using a formfitting chamber. The design of the chamber was based on tadpole morphology and tested using in vivo time-lapse imaging of neuronal structures. Physical dimensions of Stage 49 Xenopus laevis were used to determine the spatial requirements. Tadpoles with an overall body length of 11.0 ± 0.5 mm have head diameters of 3.3 ± 0.2 mm, and eyes are offset by 17.4 ± 2.5 degrees from the transverse axis. Xenopus laevis brain neurons fit within a volume of 100 microns-cubed. Thus, we set the minimum FOV, ($S_X$ and $S_Y$) and the axial requirements, $S_z$ to ensure that the neurons will fit into a volume with sides of at least 100 μm. The stimulation chamber was created by printing a negative mold using polyactide (PLA) using a 3D-printer (Creatr Dual Extruder, Leapfrog), and filling the mold with polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning). The head of the Xenopus laevis was stabilized in the chamber using a 0.2 mm thick, square sheet of cellulose acetate secured by a 3D-printed, PLA, C-clip. The Xenopus laevis’s eye was coaxial with the normal of the visual stimulator screen. The resulting field of view of the screen available to the eye is 78 ° on the horizontal and 44 ° on the vertical plane, while being submerged with several millimeters of physiological solution. Clearance between the screen and the optical axis of the microscope allows a water immersion objective with a diameter less than or equal to 33 mm, with shank angle greater or equal to 26 ° from the aperture of the objective. Objectives this size can accommodate a maximum of 9 mm motion of the objective in X, or Y directions controlled via three manual stages.
(X_M, Y_M, and Z_M) before obstruction with the chamber or screen. A mini laser-projector (ShowWX+/PicoP, Microvision), mounted directly to the stimulation chamber, projects the stimulus image on a diffuser film (Inventables, 23114-08) that is mounted on a section of a glass slide (VWR, 16004-386) with PDMS, which is the interface between the physiological solution and the project image.
Figure 3-9
The visual stimulation chamber was designed to allow simultaneous imaging and visual stimulation by projecting red and black images to screen (diffuser film) using a laser projector (inset). The head of a tadpole is stabilized inside a small cavity that has an opening allowing the tadpole’s eye to view the screen.
3.5 Results

3.5.1 Validating the optical train

The optical train was evaluated with measurements acquiring the amount of power reaching the back aperture, the axial and lateral optical resolution, and the field-of-view of the microscope. The power of the laser was measured before and after the prism used for spatial compensation and at back aperture of the objective. The power being emitted from the Ti-Sapphire, using maximum dispersion compensation was 1.94 W out of the Ti-Sapphire laser, 1.35 W after the beam expansion, and 0.35 W maximum at the back aperture of the objective using a wavelength of 910 nm to maximize the excitation of jGCaMP7s.

The axial and lateral resolution of the microscope, using two-photon excitation at 910 nm was measured using 0.1 µm diameter green fluorospheres (F-8803, Thermo Fisher Scientific). The lateral resolution was measured to be an average of 0.34 ± 0.3 µm full-width at half maximum (FWHM, N = 9) by 1.24 ± 0.3 µm FWHM (N = 9), which were the major and minor axes over the entire FOV of the image. The difference accounts for the spatial dispersion caused by the AODs due to the varied frequencies over the scan. This value remains constant between RS and F3DS since the same frequency dwell time is used. The axial resolution was measured to be 2.5±0.7 µm.

Tracking the edges of a fluorescent standard (1951 USAF Target #57-855, Edmund Optics, Group 4 - Element 6), determined a maximum FOV using the AODs and the 60X objective to be 112 × 112 µm. Using the same standard, the FOV of the digital camera was measured to be 288 × 216 µm at a resolution of 2560 × 1920 pixels.
3.5.2 Piezo-actuator validation

The performance of the piezo-actuator was evaluated for its dynamic range and response to a step-input to determine the velocity, acceleration and settling response to define the limitations during the RS and F3DS scans. The dynamic range of the Z-actuator and 0.176 Kg payload (objective and mount, Fig. 3-10A) was measured using the vendor’s onboard parameter tuning application for piezo actuators (Aerotech EasyTune™), and found to be stable up to approximately 125 Hz (Fig. 3-10B). The maximum velocity, $V_{Z,MAX}$, of the Z-stage was experimentally determined by observing the output to a step-response by observing the settling time, $T_s$, with an error less than 0.5 μm. The piezoactuator was subjected to a step response of 100 μm (Fig. 3-10C). Under the loaded conditions, a maximum velocity of 0.043 m/s was obtained in both directions with a settling time of less than $2.1\times10^{-3}$ s. Acceleration values of less than 60,000 m/s/s were found to provide stability ensuring following errors of less than $0.5\times10^{-6}$ m.

Figure 3-10
Optical train and piezoactuator performance validation. (A) The 0.176 Kg objective assembly mounted on the Aerotech, piezo actuator, and (B) the Bode plot output following a stability test.
proved the actuator stable up to ~125 Hz. (C) The Z-stage, piezo actuator's response to a step command over 100 and 10 μm, respectively. The Piezo achieved a maximum velocity of 0.043 m/s with a following error less than ± 5 × 10⁻⁶ m, and a settling time of <5.3 × 10⁻³ s.

3.5.3 Rapid-scan position validation

The RS was validated to ensure that the X-Y position of the laser was synchronized with the position of the focal plane ensuring that the laser excited the 3D-position in space defined by the coordinates of the POI. Fluorospheres, 0.1 μm in diameter (F-8803, Thermo Fisher Scientific), were embedded and distributed within a 300 μm thick layer of Sylgard 184 providing sufficiently spaced, and immobile targets for analysis. A F3DS scan captured a volume of 112×112×125 μm (slightly greater than the volume of neurons sampled for analysis) and a total of 10 beads were drawn in the CTAK UI. The RS scan was executed and the Z-actuator provided position feedback from its capacitive sensor with an average relative error of ± 3.9 % ∆F/F₀ (N = 10 beads). The capacitive feedback from the Z-actuator was compared with the desired position of the POIs and an error of 0.375×10⁻⁶ ± 0.125×10⁻⁶ m (N = 8 motion trials of the same trajectory, 68 s per motion trial) was measured with respect to the entire motion profile with an error of 0.375×10⁻⁶ ± 0.125×10⁻⁶ m (N = 1270 measurements) on the Z-axis for all nodes.

3.5.4 Experimental validation and 4D data collection

Visually evoked calcium activity was recorded in individual Xenopus tectal neurons expressing jGCaMP7s and mCyRFP1 through single-cell electroporation and used to demonstrate the system’s capabilities of the routine. We initially validated the platform’s capability to record
stimulus-evoked calcium events with a temporal resolution necessary to be accurately registered as evoked events by automated detectors in *Xenopus* tectal neurons expressing GCaMP6m (Sakaki et al., 2018). However, upon release of the 7th generation of GCaMPs we switched to jGCaMP7s due to its superior sensitivity and longer decay time which improves the platform’s capability of detecting short duration, local calcium transients across a large number of points in a 3D space, such as is the case with synaptic inputs (Dana et al., 2019). Both the higher $\Delta F/F_0$ and slower decay rate of jGCaMP7s maximize the number of samples collected above noise, giving high confidence that transients are detected in response to a stimulus. A typical result set, shown in Fig. 3-11, shows a standard deviation projection of a neuron (Fig. 3-11A-B) for images collected during the *F3DS*. The user-drawn, computer interpolated 3D-structure is shown in Fig. 3-11C. Calcium data for each of the 609 POI collected at a rate of 3 Hz is shown for the initial time period in Fig. 3-11Ci and shows $\Delta F/F_0$ traces for each of the POI recorded representing each raw ‘line-scan’ for each POI on the neuron. As indicated immediately beneath each of the line-scans four 50ms OFF stimuli are presented at pseudorandom time points on a bright background, the background is gradually shifted from bright to dark over several seconds and then four 50ms ON stimulus are presented at pseudorandom time points. An in-house designed automated event detection system (Sakaki et al., 2018) was employed to detect the number of stimulus that evoked action potentials in this neuron (2/4 OFF stimuli, 1/1 transition shift stimulus and 0/4 ON stimuli). Four separate planar RS (i.e. no moving Z-stage, scanning is exclusively provided by the X-axis and Y-axis AODs) were performed on this same neuron using a similar experimental protocol as was used in the *F3DS* scan (Fig. 3-11D-G, Di-Gi). While the planar RS lack the comprehensive imaging capability of the *F3DS* scan, they provide greater temporal resolution, recorded at scanning rates between 204-246 Hz, each recording POIs on a single Z-plane cutting through the neuron. A
comparison of the individual traces for a section of the neuron sampled both with a 3 Hz $F3DS$ scan (Fig. 3-11H, J) and a 232 Hz planar $RS$ (Fig.3-11I, K) demonstrate the advantage of an increased sampling rate for recording stimulus-evoked calcium microdomains that are potentially synaptic.
Figure 3-11

Visually evoked tectal neural activity can be recorded across the complete dendritic arbor and soma using random access TPLSM. (A,B) A standard deviation projection image in the green (A) and red channels (B) of a Xenopus tectal neuron expressing jGCaMP7s and mCysRFP created from a F3DS stack of images is shown. (C) The user-drawn POIs tracing out the structure of the neuron. A total of 609 POIs are drawn located at 2 μm distances from their nearest neighbor. (Ci) ΔF/F₀ output from the resulting F3DS of the 609 POIs, collected at 3 Hz over a 73 second interval. Responses can be seen for four 50 ms “OFF” stimuli, a “transition shift” stimulus and four 50 ms “ON” stimuli at spaced at pseudorandom time points. White asterisks indicate evoked somatic events. (D–G) The user-drawn POIs tracing out the structure of the neuron with the colored POIs sampled using a series of planar RS (Di-Gi) collected between 204 and 246 Hz with a similar experimental protocol as used previously (C-Ci). (H–I) A series of POIs that were recorded in both a 3 Hz F3DS scan and 232 Hz planar RS. (J) The traces for the POIs recorded at 3 Hz over 73.00 s. (K) The traces for the POIs recorded at 232 Hz over 80.08 s.

To improve temporal resolution relative to the F3DS scanning method while still maintaining a dataset containing recordings from across the dendritic arbor a “Segmented Scanning” was developed. In this method, the 3D structure of the neuron (Fig. 3-12A-B) is divided into 3 compartments (Fig. 3-12C). Furthermore, the majority of interstitial nodes are excluded from scanning, with sampling only occurring at the soma, branch points, filopodia bases and filopodia tips (a total number of 324 POIs for this neuron), where previous studies demonstrate there is an enrichment in the density of synapses in these neurons (Li et al., 2011). Fluorescence data from each third is serially collected in three imaging epochs and the data from all three scans
is then combined to produce a reconstructed recording of both OFF-stimulus evoked (Fig. 3-12D) and ON stimulus evoked calcium transients (Fig. 3-12E). Fig. 3-12F-G shows the $\Delta F/F_0$ traces of series of POIs at the tips and bases of filopodia recorded using a Segmented Scan at a rate of 6 Hz (4 filopodia out of a total of 174 on the neuron), where spatially restricted (2 µm) stimulus-driven calcium currents are present at the tips of filopodia that represent synaptic activity.
Figure 3-12

Visually evoked synaptic calcium transients across the neuron can be recorded with increased temporal resolution using Segmented Scanning. (A,B) A standard deviation projection image in
the green (A) and red channels (B) of a *Xenopus* tectal neuron expressing jGCaMP7s and mCyRFP created from a F3DS stack of images is shown. (C) The user-drawn POIs tracing out the structure of the neuron with the POIs that are sampled in each sub-scan of the Segmented scan are colored and the subsequent visually evoked calcium activity is shown. A total of 324 POIs located at the soma, branch points, filopodia bases and tips are collected at a rate of 6 Hz. (D,E) The maximum of the average ΔF/F₀ evoked responses for both a series of four pseudorandom 50 ms “OFF” (D) and four 50 ms “ON” stimuli (E). (F) A series of POIs recorded corresponding to filopodia tips and bases and (G) the individual calcium traces of those POIs.

The ability of this platform to record stimulus-evoked neuronal glutamate release onto a neuron was also tested using *Xenopus* tectal neurons expressing the non-ratiometric fluorescence based glutamate sensor Super-folder-GFP-iGluSnFR-A184S (Marvin et al., 2013, 2018) through employing single-cell electroporation, with a result set shown in Fig. 3-13. Segmented Scanning using 9 OFF stimuli was performed on an individual neuron expressing the construct (Fig. 3-13A) and a total of 259 POIs were collected at a rate of 6 Hz. Active areas of glutamate release could be analyzed by calculating the stimulus-evoked response for each POI, using the average DF/F₀ change between 0 and 3 seconds after the 9 stimuli (Fig. 3-13C). Responses were identified using a matched filter algorithm (Sakaki et al., 2018) to the expected iGluSnFR dynamics (\( \tau_R = 5 \) ms, \( \tau_F = 150 \) ms) using a window size of 8 samples (Fig. 3-13D) and localized back to their position in the arbor (Fig. 3-13E). Fig. 3-13F-G show the traces of a subset of 6 POI on 4 filopodia demonstrating recordings of spatially restricted, stimulus driven glutamate release onto filopodia tips.
Figure 3-13

Visually evoked synaptic-localized glutamate transients across the neuron can be detected using Segmented Scanning. **(A)** A standard deviation projection image in the green channel of a
**Xenopus** tectal neuron expressing shown sfGFP-iGluSnFR-A184S. (B) Locations of a total of 259 POIs sampled across the soma, dendritic branch points, filopodia bases and tips. (C) Stimulus-evoked iGluSnFr $\Delta F/F_0$ response for 3 s post-stimulus at each POI, collected at a rate of 6 Hz and averaged over 9 50 ms “OFF” stimuli. (D) Transient events detected using a matched filter algorithm (Sakaki et al., 2018), identifying evoked increases that match Super-folder-GFP-iGluSnFR-A184S dynamics ($\tau_R = 5$ ms, $\tau_F = 150$ ms) and a window size of 8. (E) Locations and strengths of the responses located spatially across the arbor. (F) A series of POIs recorded correspondingly to filopodia tips and bases and (G) the individual iGluSnFr traces of those POIs.

### 3.6 Discussion

Our AOD TPLSM achieved comprehensive imaging of brain neurons while providing an open-source blueprint for the full software and hardware systems. The system successfully detected activity at subcellular resolution on regions of the neuron’s dendritic arbor and cell body. We demonstrated that that our system can capture calcium-based, fluorescence activity at 2 µm resolution at 3 Hz over an entire neuron’s dendritic arbor (Fig. 3-11). Through employing Segmented Scanning (Fig. 3-12, Fig. 3-13) this system is able to increase the sampling rate to 6 Hz while generating trace data from across all filopodia and branchpoints across the dendritic arbor. Furthermore, through the planar RS this TPLSM can provide higher temporal resolution sampling (i.e. greater than 100 Hz) in continuous sections of the neuron within single focal planes (Fig. 3-11). In conjunction, these multiple scanning modes accord users of this platform a substantial degree of flexibility in tailoring its function to their experimental design.
One of the major goals of this work was to provide a software, mechanical and electrical framework to serve as a template for comprehensive imaging, as well as for future TPLSM development. We achieved this by partitioning this system into modules with distinct areas of responsibility. The low dependency between modules allowed tasks to run independently from each other (e.g., measurement tasks such as the RS) while system maintenance/monitoring was consecutively handled. It is evident that the overhead throughout the development of the system using LVOOP/Actor Framework was initially high. However, the benefit of the system’s versatility with respect to the scalability of the platform (e.g., other measurements, additional hardware configurations, other modes of imaging) greatly overshadows the burden of the initial, implementation investment.

Our UI designs (Msmt UI and the CTAK UI) handled the system/experiment administration and seamlessly integrated the operator’s user-in-the-loop tasks into the experiment (e.g., drawing the neuron, updating POI positions). Up-to-date, F3DS and FSS information from the scanned neuron provided the user with the means to identify and maintain the relationship between the neuron’s morphology and the TPLSM system analyzing each POI extracted from the morphology. It is apparent that having a user-in-the-loop will have obvious advantages, depending on the skill of the operator (e.g., quickly identifying small cell features, tracing the neuron), and disadvantages depending on the duration of the experiment (e.g., operator fatigue and fatigue-related mistakes). However, given the current limitations of machine vision algorithms’ accuracy in localizing the topography of the neuron’s arborisation (Peng et al., 2011), we hope to develop or encourage the development of fully automated methods of identifying and maintaining the neuron’s structure and eliminate the need for the user-in-the-loop.
Our RS utilizes a hybrid AOD-piezoactuator combination to acquire calcium-related activity from POI scans in 3D by acquiring all of the POIs using a planned trajectory. The planned trajectory synchronizes the motion of the piezoactuator with the laser deflection angle of the AODs on the X-Y plane. Using this method, we have achieved comprehensive imaging rates up to 4 Hz and have sustained this method over durations of 68 seconds with measured errors of no more than $0.375 \times 10^{-6} \pm 0.125 \times 10^{-6}$ m throughout the duration of the experimental trials.

We acknowledge that an obvious bottleneck exists at the piezoactuator as a result of the mechanical motion. However, we believe we achieved the overarching goal of developing a versatile system for comprehensive imaging, which can be optimized through further design improvements and minimal effort, as current research has demonstrated that incorporating compact remote focusing technologies with acousto-optics has become feasible (Nadella et al., 2016). Furthermore, the simplicity of the design requires only basic skills for implementation to achieve initial imaging as opposed to more complex systems where remote focus designs are used or where optical trains are designed and dedicated to compensating for the dispersion of AODs.

In this work, we described an open-source, rapid-access, two-photon laser-scanning system for comprehensive sampling and analysis of neuronal activity. We demonstrated a system capable of extracting and creating a structural representation of a neuron and converting that structure into points-of-interest (POIs) to saturate sampling of activity in the cell body and throughout the entire dendritic arbor. Our system, using the list of POIs, creates a schedule for rapid-scanning, and coordinates acousto-optics and a high-speed, piezo linear-actuator to position a laser on each POI for rapid-scanning. We devised a 3D-printed chamber, and visual stimulator system to provide controlled sensory stimuli to awake immobilized animals. In conjunction, this enables us to record visually-evoked responses across the complete dendritic arbor and soma of an individual neuron.
with in an intact, awake developing brain. This is a novel capability and has broader appeal for the developmental neuroscience community, in particular allowing for the investigation of experience-driven neuronal growth and development of tuning.

Our versatile, open-source object-oriented, system software architecture was built using Actor Framework and LabVIEW ‘G’ architecture using a powerful, established and commercially maintained API. This system architecture can be easily adapted for further experimental design as well as easily adapting the routines for other architectures, lending itself for future rapid prototyping as well as more mature designs in either research or for industrial purposes.

Our validated AOD-based random-access TPLSM with software is specifically designed to capture calcium biosensor fluorescence of neural activity throughout a neuron’s entire dendritic arbor and soma in the intact and awake brain. Together, this hardware and software platform is capable of comprehensive imaging, required for understanding synaptic integration and neural encoding.

Additional documentation for this platform and instructions for further access to relevant resources is located at the UBC Dynamic Brain Circuits in Health and Disease GitHub page: https://github.com/ubcbraincircuits
Chapter 4: Sensory experience modifies developing neurons

4.1 Introduction: Investigating neural plasticity in an in vivo developing model

Previous research in the Haas lab has investigated the relationship between stimulus-driven structural and functional plasticity during early brain circuit development in vivo (Dunfield & Haas, 2009). Through utilizing the transparent albino Xenopus tadpole visual system as an accessible model for investigating visual stimuli-driven plasticity within the developing brain, it has been found that specific patterns of visual stimuli can cause lasting, NMDAR-dependent changes in the visual-evoked responses of neurons in the optic tectum similar to classic LTP and LTD experiments (Dunfield & Haas, 2009). These findings suggest the existence of Hebbian (Hebb, 1949) based experience-driven plasticity in a developing model. More recently, preliminary studies in the Haas lab have shown that when a neuron becomes wired into a sensory circuit in vivo, there is an experience-driven net increase in dendritic filopodia pruning, while calcium signals from regions of the arbor that become tuned to the stimuli also show clustering (Podgorski et al., 2021). There is further research that demonstrates that activity can induce filopodia growth or stabilization in vitro (Lohmann et al., 2002). Of note, there is an enrichment of synapses at the tips of filopodia in Xenopus tectal neurons (Li et al., 2011).

Experience-based tuning of developing neurons in the visual system has been demonstrated in in vivo experiments by recording evoked neuronal somatic responses corresponding to action potential firing and measuring how they change in response to plasticity inducing visual training (Dunfield & Haas, 2009). However, it remains unknown how these training experiences impact synaptic functional plasticity and concordant dendritic growth plasticity in these neurons, and the relation between synaptic/dendritic plasticity to the functional plasticity of somatic output. Little
research has focused on such questions due to limitations in conventional microscopy, particularly challenges in sampling sensory-evoked activity across the complete three-dimensional (3D) structure of large and complex brain neurons in vivo. Such experiments require high spatial and temporal resolution to track synaptic activity across the dendritic arbor as well as action potential outputs, correlated with relatively slower imaging of structural changes associated with dendritic growth and synaptogenesis. The slow speed of imaging with conventional laser-scanning two-photon microscopy has restricted in vivo investigations of experience-driven plasticity to investigations of effect only on structural growth, or activity in two-dimensional (2D) planes (El-Boustani et al., 2018; Kerlin et al., 2019; Makino & Malinow, 2011; Scholl et al., 2017). The combination of optimized calcium sensors and a fast point-scanning two photon microscope developed by myself and other members of the Kurt Haas laboratory unlocked a unique capability for me to address these outstanding challenges in the field. I was able to record both calcium signals and structure of complete dendritic arbors of individual neurons in vivo while the awake animal is receiving controlled visual stimuli. This allowed me to record visually evoked activity across the entire structure of the neuron at hundreds to thousands of points at rates of 6-10Hz sufficient for tracking calcium transients. In conjunction with employing our event detection software to track somatic firing (Sakaki et al., 2018), this process provides a comprehensive understanding of all synaptic calcium activity across the entire neuron while also being able to correlate these activity patterns with changes in dendritic structure such as filopodia additions or subtractions (Fig. 4-1). Using these technologies, I am able to investigate how neurons responded to a plasticity-inducing stimulus, spaced training and ultimately generate an understanding of how developing neurons are modified by experience.
I hypothesized that visual training improves encoding properties of developing brain neurons through coordinated structural and functional changes across the dendritic arbor. Structural changes, including additions and pruning of dendritic filopodia containing synaptic inputs is correlated to local evoked calcium transients, resulting in clustering of filopodial synaptic inputs responsive to the entrained stimulus. Simple rules of calcium-directed local dendritic growth dynamics, synapse selection and topography underlie the formation of neural structure and connectivity that promotes experience-driven single-neuron encoding improvement.
Figure 4-1

*In vivo* recording of changes in structure and function of tectal neurons. (A-C) A 3D stack of the neuron is made (A), a skeleton of points of interest is drawn (B) and functional data is recorded at those points of interest (C). (D-F) Post spaced-training to OFF stimulus, another image stack is created (D), the skeleton is adjusted to compensate for drift of the neuron and also for structural changes, with filopodia additions and subtractions being tracked (E) and then functional data is recorded from the new skeleton (F).

4.2 Results
4.2.1 Tectal neurons can be separated into groups based on activity and the different activity groups have distinct response patterns to plasticity inducing visual stimuli

My first experimental goal is to establish the presence of distinct sub-groups of visually responsive tectal neurons that are tuned to different visual stimuli and to establish if these different response properties will result in differences in how their tuning and growth behaviours respond to a specific visual stimulus. I aimed to determine whether tectal neurons can be divided into discreet groups based on their somatic, action potential responses to brief 50 ms ON and OFF visual stimuli and to investigate if their baseline response patterns to this stimulus will determine their response to strong OFF stimuli training. In order to test this I employed a visual training paradigm called “Spaced Training” that has been previously established in the Haas lab to induce LTP in populations of tectal neurons in vivo (Dunfield & Haas, 2009). Spaced training involves a series of alternating 5min epochs of high-frequency bursts of 50 ms OFF stimuli, with 5min of ON light epochs. Spaced training has been shown to induce either short- or long-lasting potentiation of OFF-evoked somatic responses in ~50% of tectal neurons, with varied responses due to each neuron’s somatic receptive field tuning to OFF or ON, and their metaplastic state (Dunfield & Haas, 2009). I employed this established somatic plasticity-induction training stimulus to examine effects on synaptic responses and dendritic growth. First, I tested the somatic tuning of individually labeled jGCaMP7s-P2A-mCyRFP1 tectal neurons, and determined how spaced training to OFF stimuli impacts dendritic OFF- and ON-evoked dendritic responses and growth.

I performed a total of 32 spaced training experiments and sorted neurons based on their responsiveness to the entrained stimulus, OFF. I found that 3 neurons showed long-term potentiation of AP outputs, 1 neuron that showed short-term potentiation, 10 that were responsive but did not potentiate, 6 that became responsive and 12 were non-responsive. The neurons were
also grouped based on whether or not they demonstrated dendritic potentiation. I first wanted to analyze whether a neuron’s maturation state would predict its plasticity response to spaced training. To do this I analyzed the interstitial filopodial density and the total dendritic branch length (TDBL) of each neuron. Both the average TDBL (Fig. 4-2A and C) and interstitial filopodia density (Fig. 4-2B and D) were not significantly different regardless of the action potential or dendritic tuning of the neuron. This finding demonstrates that neither the initial responsiveness of a neuron to the training stimulus, nor its plasticity in response to training was dependent on its baseline arbor size or density.
Figure 4-2

Neuron maturation state does not predict responsivity or plasticity in response to spaced training. Neurons expressing jGCaMP7s-P2A-mCyRFP1 underwent spaced training to OFF stimulus and were grouped based on their action-potential plasticity and their dendritic plasticity.
The average total dendritic branch length (A and C) and interstitial filopodia density (B and D) were recorded for each group. (A-B) Tukey’s HST, (C-D) t-test. Error bars indicate ± SEM.

It is an open question what the mechanism of experience induced potentiation is in these neurons. Does the degree of synaptic input correlate to the action potential output? Would strengthening of output in response to training be accompanied with a strengthening of total input? To investigate these questions, I grouped neurons both by their initial action potential response properties to OFF stimulus and based on whether or not they showed OFF-evoked action potential potentiation in response to spaced training (Fig. 4-3). I found that neurons that fired action potentials to OFF stimulus also showed a higher proportion of their filopodia that were responsive to OFF stimulus compared to neurons that did not fire to OFF (Fig. 4-3A), and that this relationship between input strength and output strength was consistent post-training (Fig. 4-3B). Furthermore, in neurons that showed an OFF output potentiation during training, there was a corresponding input potentiation that was specific to the OFF response (Fig. 4-3C-D). Taken together, these findings demonstrate that action potential output is linked to the strength of dendritic input and that strengthening of action potential output corresponds with a strengthening of dendritic input.
Figure 4-3

Action potential activity correlates with dendritic activity. (A-B) Neurons were grouped based on whether they were somatically responsive to OFF stimulus before (A) and after spaced training to OFF (B) and the average percentage of filopodia that responded OFF stimulus was analyzed. (A) *: P<0.05, t-test. (B) *: P<0.05, **: p<0.01, t-test. (C-D) Neurons were grouped based on whether they showed somatic potentiation to OFF stimulus during spaced training to OFF and
the average percentage of filopodia that responded OFF stimulus (C) or ON stimulus (D) was analyzed. *: P<0.05, t-test. Error bars indicate ± SEM.

4.2.2 Spaced training modifies dendritic arbor growth and remodelling in a context sensitive pattern

It is known that in developing neurons, the dendritic arbor is a highly dynamic structure and that this cytoskeletal remodelling is linked with synapses rapidly being assembled and disassembled (El-Boustani et al., 2018; Haas et al., 2006; Hossain et al., 2012; Sin et al., 2002). We know that dendritic arbor growth is linked to synapse formation (Niell et al., 2004) and is mediated by activity (Espinosa et al., 2009; Haas et al., 2006). Thus, it is an important question to ask how this structural remodelling is organized based on the activity pattern of a particular neuron. Consequently, I analyzed the change in filopodial density, addition and subtraction rates of each neuron grouped by their action potential or dendritic tuning in response to spaced training (Fig. 4-4). In general, all groups of neurons showed an increase in the number of additions in response to training, and this was significant for the group that were not responsive to that training (Fig. 4-4 A-B), whereas neurons that underwent short-term or long term potentiation showed elevated rates of subtractions (Fig. 4-4 C-D) and neurons that became responsive to OFF stimulus showed a post-training increase in filopodial density.
Figure 4-4

Plasticity in activity is linked to patterns of structural changes. **(A-B)** Average interstitial filopodial addition rates relative to baseline. *: P<0.05, **: p<0.01, ANOVA with a post hoc Dunnet’s test.  
**(C-D)** Average interstitial filopodial subtraction rates relative to baseline. *: P<0.05, ANOVA with a post hoc Dunnet’s test.  
**(E-F)** Average interstitial filopodial density. *: P<0.05, ANOVA with a post hoc Dunnet’s test. Error bars indicate ± SEM.
Previous research has found that there is patterned synaptic topography at the microcircuit level (N. Takahashi, 2019), with \textit{in vivo} experimental evidence showing patterned synaptic input in the visual cortex of both ferrets (Scholl et al., 2017; Wilson et al., 2016) and in mice (Iacaruso et al., 2017). However, as this research was performed in mature animals, it remains unknown how this patterned topography arises during development. To investigate this, I analyzed whether space-training-induced filopodial additions were clustered closer to filopodia responsive to the trained stimulus. For neurons that underwent short-term potentiation or long-term potentiation of action potential output, filopodial additions occurring during training showed increased clustering specifically to OFF responding filopodia relative to the additions that arose prior to training (Fig. 4-5A-B), indicating that the signaling driving the clustered additions is associated with local responses to the trained stimulus. When the neurons were grouped by their dendritic plasticity, neurons that potentiated to OFF stimulus showed clustering of new additions to OFF responding filopodia but not ON responding filopodia both during training and at 30 minutes post-training, while the group of neurons that did not dendritically potentiate did not show significant training induced clustering of additions to OFF or ON responding filopodia (Fig. 4-5C-D). For all plasticity groups subtractions did not show clustering to either OFF or ON responding filopodia at any timepoint (Fig. 4-5E-H). These finding demonstrate that the clustered patterns of new additions is associated with localized hot-spots of synaptic activity driven specifically by the entrained stimulus and that this clustering pattern is particularly seen in neurons that show potentiation to the training stimulus.
Spaced training modifies filopodial survival and responsiveness

There is existing research that demonstrates that spaced training induces filopodial stabilization and PSD-95 aggregation, which indicates synapse formation (S. X. Chen et al., 2010; Niell et al., 2004). Consequently, I wanted to investigate if these clustered additions arising during training would show an increase in survival and responsivity to the entrained stimulus. When all neurons were grouped together, additions that arose during training that were clustered to an existing OFF-responsive filopodia showed increased survival rates over 1 hour compared to non-clustered additions (Fig. 4-6A). Furthermore, when the neurons were grouped by both output plasticity and input plasticity, it was specifically the groups that potentiated to OFF (S/LTP, become responsive, dendritically potentiate) that showed this enhanced survival of OFF clustered additions (Fig. 4-6 B-D), while filopodial additions in stable and non-responsive neurons did not show any difference in survival rates between clustered or unclustered additions. Interestingly, when the same filopodial additions were clustered to the nearest existing ON-responsive filopodium there was similar but generally weaker patterns, with a significantly higher survival
rate for filopodia that are clustered for all neurons grouped together and in particular the S/LTP and non-responsive groups (Fig. 4-6G-L). I also found that a new addition arising during training that is clustered to an OFF-responsive filopodium would be more likely to itself be responsive to OFF stimulus over the next hour then an unclustered filopodium when the neurons were all grouped together and in the subset that dendritically potentiated (Fig. 4-7A-F) and the probability of these filopodia responding to ON stimulus was not related to whether they were clustered to a existing ON responding filopodium or not (Fig. 4-7G-L). OFF responsive additions were also more likely to survive over the next 1 hour then non-responsive additions (Fig. 4-8A), particularly for neurons that showed potentiation of inputs or output to OFF (Fig. 4-8 B-F). Similar results were seen when categorizing filopodial additions by their ON responsivity for when all neurons were grouped together and specifically in the group that became responsive to OFF (Fig. 4-8G-L).

In summary, filopodial additions that are clustered to OFF responding filopodia are more likely to survive and become OFF responsive themselves and that those that are indeed OFF responsive are more likely to survive. These results are similar to would be expected from new filopodia that are contextually linked to the training response becoming integrated, compared to non-clustered, non-responsive additions arising during the same time-point. This is reinforced by the fact that these results are particularly pronounced in neurons that show potentiation to the trained stimulus. Of note, the fact that there is a similar but weaker pattern of enhanced survival and responsivity when looking at the ON response, suggests some connection between these two pathways and that OFF training has a secondary effect on the ON circuit. Overall, these findings are indicative of these new-born filopodia becoming integrated into the entrained circuit, where filopodia that are clustered and responsive to the relevant stimulus survive while those that are not are pruned (Fig. 4-9).
Figure 4-6

Clustering of filopodia added during spaced training to existing responsive filopodia promotes survival. (A-F) Neurons were grouped by their plasticity and the survival of additions arising during spaced training to OFF that were clustered or not clustered to the nearest OFF responding filopodia was tracked. (G-L) Neurons were grouped by their plasticity and the survival of additions arising during spaced training to OFF that were clustered or not clustered to the nearest ON responding filopodia was tracked. *: P<0.05, **:p<0.01, ***:p<0.001, Chi-squared test.
Figure 4-7
Filopodial additions arising during spaced training that are clustered to existing OFF responsive filopodia are more likely to be themselves responsive to OFF. (A-F) Neurons were grouped by their plasticity and the responsivity of additions arising during spaced training to OFF stimulus that were clustered or not clustered to the nearest OFF responding filopodia was tracked. (G-L) Neurons were grouped by their plasticity and the responsivity of additions arising during spaced training to OFF stimulus that were clustered or not clustered to the nearest ON responding filopodia was tracked. *: P<0.05, Chi-squared test.
Figure 4-8
Filopodial additions arising during training that are responsive are more likely to survive. (A-F)
Neurons were grouped by their plasticity and the survival of additions arising during spaced training to OFF that were responsive or not responsive to OFF stimulus was tracked. (G-L)
Neurons were grouped by their plasticity and the survival of additions arising during spaced training to OFF that were responsive or not responsive to ON stimulus was tracked. *: P<0.05, Chi-squared test.

Figure 4-9
Dendrite growth behavior directed by local calcium transients promotes clustering of tuned synapses. Peak OFF-evoked responses at all imaging sites of a neuron labeled with jGCaMP7s-P2A-mCyRFP1 recorded in vivo before (left) and after (right) Spaced Training. Inset shows
filopodial additions, subtractions and responsive filopodia for the highlighted region of the neuron.

The next step was to determine if this patterned, contextually sensitive growth and survival of filopodia arising during spaced training was mediated by intracellular or extracellular cues. To investigate this, I looked at additions that arose during training that were clustered to existing OFF responsive filopodia by extracellular distance, but not intracellular distance and compared them with either intracellularly clustered filopodia or unclustered filopodia (Fig. 4-7). I found that intracellularly clustered filopodia added during training were significantly more likely to survive over the next hour then filopodial additions that extracellularly but not intracellularly clustered (Fig 4. 10B) and that these extracellularly only clustered filopodia did not have a significantly different survival rate then unclustered filopodia (Fig. 4-10C). These results demonstrate that the enhanced survival of clustered additions during training are dependent on intracellular distance not extracellular distance, indicating that patterned growth is mediated by intracellular signaling.
Intracellular distance from OFF responding synapses predicts survival of additions that arise during spaced training. (A) Schematic of measuring extracellular and intracellular distance between two filopodia in a dendritic arbor. Insert is an example of extracellularly clustered but not intracellularly clustered pair of filopodia compared to a pair of filopodia that are both intracellularly and extracellularly clustered. (B) Survival of additions arising during training that were both intracellularly and extracellularly clustered compared to those that were only extracellularly clustered. *: P<0.05, **: P<0.01, Chi-squared test. (C) Survival of additions arising
during training that were only extracellularly clustered compared to those that are not clustered. Chi-squared test.

4.2.4 Potentiation of action potential output is linked to potentiation of synaptic inputs

An open area in my dataset was to characterize the linkage between the level of dendritic input potentiation and action potential output potentiation. I wanted to determine if neurons that showed a potentiation of output would also show a potentiation of inputs and characterize whether synapses showed both an increase in average amplitude and a decrease in failure rates. To do this I analyzed the tips of filopodia of neurons before, during and after spaced training, where it is known that there is a high density of synapses in Xenopus tectal neurons (Li et al., 2011). In five neurons that showed potentiation of outputs to OFF stimulus in response to spaced training to OFF, I found that the average evoked response to OFF stimulus at filopodia tips also increased post training, and this response seemed to be linked to an increase in firing probability (Fig. 4-11 A-C). In addition, the filopodial tips showed a similar potentiation to ON stimulus (Fig. 4-11 D-F). This was in large part due to some filopodia showing responses to both OFF and ON stimulus (Fig. 4-12), and strengthening to the untrained stimulus as well. When specifically looking at additions arising at each timepoint, I found that additions subsequent to training showed an increased responsivity to both OFF (Fig. 4-13 A-C) and ON stimulus (Fig. 4-13 D-F) relative to existing filopodia and this was due to both an increase in the evoked amplitude of the response and due to an increase in the response rate to the stimulus. The filopodia that were subtracted during and subsequent to training were significantly less responsive to OFF stimulus then non-subtracted filopodia (Fig. 4-14 A-C) and this was specific to the OFF stimulus (Fig. 4-14 D-F). Together these results are indicative of neurons that are become wired to entrained stimulus, with existing
filopodia showing more responsivity to the training stimulus, as well as new additions being especially responsive to the training stimulus and the preferential subtraction of filopodia that were less responsive to the training stimulus. Consequently, it is clear that neurons that show a potentiation of output to a stimulus also demonstrate a potentiation of inputs.
Figure 4-11

Potentiation of output is correlated with potentiation of input. (A-C) Average OFF response at all filopodial tips for five neurons that showed potentiation of output to OFF stimulus in response to spaced training to OFF. (A) The average evoked filopodial amplitude after each OFF stimulus. (B) The average evoked filopodial amplitude after each OFF stimulus when failures are excluded.
(C) The average evoked filopodial probability of firing after each OFF. *: P<0.05, **:p<0.01, ***:p<0.001, ANOVA with a post hoc Dunnet’s test. Error bars indicate ± SEM. (D-F) Average ON response at all filopodial tips for five neurons that showed potentiation of output to OFF stimulus in response to spaced training to OFF. (D) The average evoked filopodial amplitude after each ON stimulus. (E) The average evoked filopodial amplitude after each ON stimulus when failures are excluded. (F) The average evoked filopodial probability of firing after each ON. *: P<0.05, **:p<0.01, ***:p<0.001, ANOVA with a post hoc Dunnet’s test. Error bars indicate ± SEM.
Figure 4-12
Stimulus evoked calcium transients can be detected across the dendritic arbor and spatially isolated. 

(A) A neuron labeled with jGCaMP7s-P2A-mCyRFP1 is skeletonized and divided in thirds and sampled independently at with a battery of 4 OFF stimulus and 4 ON stimulus and activity is recorded from across the dendritic arbor. (B) A branch in the dendritic arbor of the neuron is highlighted and the traces recorded from three spatially clustered filopodia presented.
Figure 4-13

Potentiation of output is correlated with increased synaptic strength in additions post-training. (A-C) Average OFF response at the filopodial tips of all additions for five neurons that showed potentiation of output to OFF stimulus in response to spaced training to OFF. (A) The average evoked filopodial amplitude after each OFF stimulus. (B) The average evoked filopodial amplitude after each OFF stimulus when failures are excluded. (C) The average evoked filopodial probability of firing after each OFF. *: P<0.05, **:p<0.01, ***:p<0.001, ****:p<0.0001, t-test. Error bars indicate ± SEM. (D-F) Average ON response at the filopodial tips of all additions for five neurons
that showed potentiation of output to OFF stimulus in response to spaced training to OFF. (D) The average evoked filopodial amplitude after each ON stimulus. (E) The average evoked filopodial amplitude after each ON stimulus when failures are excluded. (F) The average evoked filopodial probability of firing after each ON. *: P<0.05, **:p<0.01, ***:p<0.001, ****:p<0.0001, t-test. Error bars indicate ± SEM.
Figure 4-14

Potentiation of output is correlated with pruning of less responsive filopodia. (A-C) Average OFF response at the filopodial tips of all subtractions the epoch prior to when they were subtracted for five neurons that showed potentiation of output to OFF stimulus in response to spaced training to OFF. (A) The average evoked filopodial amplitude after each OFF stimulus. (B) The average evoked filopodial amplitude after each OFF stimulus when failures are excluded. (C) The average evoked filopodial probability of firing after each OFF. *: P<0.05, **:p<0.01, ***:p<0.001,
t-test. Error bars indicate ± SEM. (D-F) Average ON response at the filopodial tips of all subtractions the epoch prior to when they were substracted for five neurons that showed potentiation of output to OFF stimulus in response to spaced training to OFF. (D) The average evoked filopodial amplitude after each ON stimulus. (E) The average evoked filopodial amplitude after each ON stimulus when failures are excluded. (F) The average evoked filopodial probability of firing after each ON. T-test. Error bars indicate ± SEM.

4.2.5 Glutamatergic input is linked to patterned structural plasticity

In addition to excitatory GABA at early developmental stages (Akerman & Cline, 2006), glutamate serves as the primary excitatory neurotransmitter in the *Xenopus* visual system. Glutamatergic input has been shown to be essential in directing activity-dependent dendritic arbor growth and complexity in tectal neurons, acting through synapse formation, stabilization and loss (Haas et al., 2006). Synaptic activity-driven changes in dendritic arbor remodeling have been shown to be dependent on two major types of glutamatergic receptors, NMDAR (Sin et al., 2002) and AMPAR (Haas et al., 2006).

Now that I had characterized input plasticity patterns postsynaptically through tracking evoked calcium activity, I wanted to analyze how visual experience shapes synaptic tuning by directly investigating the role of glutamatergic input on directing dendritic growth, focusing and whether there is a correlation between sensory-evoked glutamatergic signals and new filopodial additions, and other patterned growth dynamics. To do this I expressed the second generation of the green fluorescence intensity-based glutamate sensor, iGluSnFR. iGluSnFR is an artificially generated glutamate receptor derived from the glutamate binding protein, GltI found in *E. coli* conjugated to a circularly permuted (cp) GFP (Marvin et al., 2013), with the second generation of
variants using the substantially brighter circularly permuted super-folder (cp-sf) GFP (Marvin et al., 2018). When glutamate binds to GltI it changes its conformation resulting in a substantial increase in its fluorescence (Marvin et al., 2013, 2018). iGluSnFR has previously been validated as a sensor of synaptically-released glutamate in vivo in zebrafish, C. elegans and mouse models (Marvin et al., 2013). By expressing iGluSnFr in single Xenopus tectal neurons and imaging with our AOD-RAMP two-photon microscope before, during and after spaced training to OFF stimulus, it is possible to record sensory-evoked upstream glutamatergic input across the complete dendritic arbor and track both changes in glutamate release onto the arbor and structural changes in the arbor.

I found that additions occurring during spaced training to OFF stimulus were on average significantly closer to a pre-existing OFF responding filopodia then additions that occurred prior to training (Fig. 4-15A). Furthermore, these additions that arose during training were significantly more clustered then a Monte Carlo modelled control and this significant difference in clustering was preserved when directly comparing the pre-training additions to the during training additions. Filopodial subtractions, like the results seen with jGCaMP7s labeled cells, appeared to be no more or less clustered before and after training (Fig. 4-15B). This indicates that training induced clustered growth is associated with stimulus-evoked glutamatergic input to the entrained stimulus. I also found that glutamatergic input also was important to determining filopodial survival. Additions arising during spaced training stimuli that were clustered to existing stable filopodia receiving stimulus-evoked glutamate were more likely to survive over the subsequent hour than additions that were not clustered (Fig. 4-15C). As well, additions arising during OFF training that receive stimulus-evoked glutamate were more likely to survive than additions that do not (Fig. 4-15D). Together, these results demonstrate that stimulus-evoked glutamatergic input acts to
selectively protect new filopodia from being pruned and provide additional evidence that filopodia that arise during training are being wired into the entrained circuit. However, this still left unanswered the question of whether glutamatergic input itself is modified across the dendritic arbor in response to visual training. The previous experiments demonstrating training-induced calcium transient changes across the dendritic arbor could be due to either post-synaptic plasticity, such as an increase in AMPA receptor trafficking, or presynaptic plasticity, such as a change in the amount of glutamate released at a synapse in response to a stimulus or the probability of glutamate release at that synapse in response to the stimulus. To test this, I analyzed the glutamatergic input at the tips of the dendritic filopodia of the six neurons labeled with iGluSnFR before during and after spaced training. I found that the average evoked glutamatergic input in response to OFF stimulus at filopodia tips also did not change in response to spaced training, either in percent firing rate or amplitude (Fig. 4-16 A-C). When filopodia were divided based on their motility, additions showed no significant difference from non-added filopodia at the same time-point for either overall average evoked glutamatergic input, or probability of firing, but average evoked amplitude was higher in additions during training then for non-added filopodia (Fig. 4-16 D-F). Filopodia that were subtracted did not have a significant difference in their overall average evoked glutamatergic input the epoch before they were subtracted comparted to non-subtracted filopodia at the same timepoint (Fig. 4-16 G). However, post-training there was a difference between subtracted and non-subtracted filopodia, with subtracted filopodia showing less frequent glutamatergic inputs in response to OFF stimulus but overall larger amplitudes (Fig. 4-16 H-I). This difference could be due to a selective elimination in smaller evoked glutamatergic transients but not larger ones in filopodia that were subsequently subtracted. When the change in synaptic weights of filopodia subsequent to spaced training in both the jGCaMP7s and iGlusnfr labeled
neurons were compared, both groups showed subsets of filopodia that strengthened and weakened in response to training. However, there was a significant difference in the proportion of filopodia that demonstrated changes in evoked synaptic amplitude between the two groups (Fig. 4-17 A-C). This demonstrates that both evoked glutamatergic input and the resultant synaptically driven calcium transients are modified by visual experience. For both evoked glutamatergic input and evoked calcium response, I found that filopodia that potentiate and have a nearest neighbour that also potentiates show a significantly higher evoked amplitude to off stimulus, then filopodia who have a nearest neighbour which does not potentiate. This provides evidence that synapses that strengthen in response to training are spatially organized.
Glutamatergic input modifies filopodial growth and survival. (A-B) Average distance between an added (A) and subtracted (B) filopodia and the nearest OFF responding filopodia for six neurons expressing iGluSnFR before, during and after spaced training. *: P<0.05, ANOVA with a post hoc Dunnet’s test. Error bars indicate ± SEM. (C-D) The survival (C) and responsiveness (D) of additions arising during spaced training to OFF that were clustered or not clustered to the nearest
OFF responding filopodia was tracked. *: P<0.05, Chi-squared test. (E) The survival of additions arising during spaced training to OFF that were responsive or not responsive to OFF stimulus was tracked. *: P<0.05, Chi-squared test.
Figure 4-16
Glutamatergic inputs are not globally modified in response to spaced training. (A-C) Average OFF response at all filopodial tips for six neurons expressing iGluSnFR. (A) The average evoked filopodial amplitude after each OFF stimulus. (B) The average evoked filopodial amplitude after each OFF stimulus when failures are excluded. (C) The average evoked filopodial firing rate after each OFF stimulus. ANOVA with a post hoc Dunnet’s test. Error bars indicate ± SEM. (D-F) Average OFF response at the filopodial tips of all additions. (D) The average evoked filopodial amplitude after each OFF stimulus. (E) The average evoked filopodial amplitude after each OFF stimulus when failures are excluded. (F) The average evoked filopodial firing probability after each OFF stimulus. *: P<0.05, t-test. Error bars indicate ± SEM. (G-I) Average OFF response at the filopodial tips of all subtractions the epoch prior to when they were subtracted. (G) The average evoked filopodial amplitude after each OFF stimulus. (H) The average evoked filopodial amplitude after each OFF stimulus when failures are excluded. (I) The average evoked filopodial firing probability after each OFF stimulus. *: P<0.05, **: P<0.01, t-test. Error bars indicate ± SEM.
Figure 4-17
Spaced training strengthens synapses both presynaptically and postsynaptically. (A) The change in OFF evoked fluorescence amplitude recorded at the tips of all filopodia that exist prior to spaced training and are stable for the next 1.5 hours for a neuron labeled with jGCaMP7s-P2A-mCyRFP1 (top) and iGlusnFR (bottom). The colour of the points represents the change in DF/F0 relative to pretraining levels. (B) Raster plot representation of the potentiation of filopodia tip potentiation changes in (A) for the jGCaMP7s-P2A-mCyRFP1 (left) and iGlusnFR (right) labeled neurons. (C) Comparison of the proportion of filopodia that show a potentiation of OFF evoked activity at each timepoint during and post spaced training for five jGCaMP7s-P2A-mCyRFP1 labeled neurons and six iGlusnFR labeled neurons. *: P<0.05, **:p<0.01, ***:p<0.001, Chi-squared test. (D-E) Grouping all stable filopodia with their nearest neighbour, sorting these pairs into groups based on whether neither, one or both filopodia potentiate to OFF stimulus and averaging the evoked amplitudes at 60 minutes post training. Tukey’s HST, *: p<0.05, **:p<0.01, ***:p<0.001. Error bars indicate ± SEM.

4.3 Discussion
This research demonstrates that growth and pruning of developing dendrites is regulated by sensory experience in an activity-dependent manner based on the relationship between an environmental stimulus and each neuron’s tuning. By imaging action potential activity, I demonstrate that experience-driven potentiation of output is associated with increased pruning, and my fast-imaging single-neuron experiments show that pruning is selective for filopodia unresponsive to the training stimulus. This supports a model in which neurons well integrated
within a sensory circuit become further functionally specialized during refinement by pruning connections to less-active inputs. In contrast, neurons can shift their spike tuning to a trained stimulus by increased growth and formation of new synaptic contacts with the driven circuit until they develop somatic responses to that sensory signal.

Sensory training-induced pruning was not observed in newly differentiated tectal neurons undergoing initial stages of dendritic arbor elaboration and synaptogenesis (S. X. Chen et al., 2012). Instead, immature tectal neurons uniformly exhibited enhanced growth in response to sensory training. During early circuit formation, sensory input without coincident firing may promote loose integration into functional circuits for the establishment of initial receptive field properties (Andreae & Burrone, 2015). Thus, early stimulus-driven growth mechanisms may be similar to those exhibited by more mature neurons shifting their spike tuning, and stimulus-driven pruning may emerge when neurons establish robust coordinated spike tuning.

A leading question in understanding neuronal circuit function is whether synaptic distribution across dendritic arbors is patterned, and if so, how such synaptic topography arises during neural circuit formation. Here, I find clustering of synapses with similar response properties to visual stimuli. By combining full-neuron imaging of neural activity and detailed tracking of structural plasticity, I find rules based on local and global activity that drives synapse clustering based on tuning. I find that the locations of filopodial additions are predicted by both local patterns of glutamatergic input and of calcium transients in the synaptic compartment. In response to training, newly formed filopodia that receive input from the salient stimulus are selectively preserved. Contrastingly, new filopodia are subtracted when stimuli evoke small calcium transients in a filopodium tip relative to the calcium transients in the local dendritic shaft. These conditions likely reflect filopodia not receiving synaptic input when dendritic shaft
calcium is high, driven by strong synaptic input and/or back-propagating action potentials (Spruston et al., 1995).

The broad distribution of backpropagating action potentials across the dendritic arbor, and the expected disparate connectivity of developing neurons to a diversity of upstream inputs are consistent with my observation that subtractions are not patterned by activity. In contrast, morphological stabilization occurs in both filopodia that receive glutamatergic input and those with larger calcium transients at their tip than their base. Critically, both filopodia that receive stimulus driven glutamate and produce a calcium response promote the addition of new filopodia in close proximity that respond to the same stimulus, leading to spatial clustering of synapses tuned to a given stimulus. Furthermore these new filopodia that are clustered are preferentially preserved from pruning. The net result of this is that morphological change in the dendritic arbor is directed towards a structural remodeling where filopodia receiving glutamatergic input and producing calcium responses to the prescient environmental stimulus act as hotspots of for new filopodia and these new filopodia are preferentially stabilized while non-responsive and non-clustered filopodia become destabilized. This is indicative of a neuron undergoing refinement as it becomes wired into a circuit.

In mature circuits, activity-driven formation of new synapses has been found to be clustered (Frank et al., 2018; Fu et al., 2012; Kleindienst et al., 2011; Takahashi et al., 2012), but serial mapping of synapse tuning in vivo has found evidence for spatial clustering of synapses tuned to the same input in some neurons, but not others (Chen et al., 2013; Chen et al., 2011; De Roo et al., 2008; Druckmann et al., 2014; Frank et al., 2018; Iacaruso et al., 2017; Jia et al., 2010; Ju et al., 2020; Kleindienst et al., 2011; Lee et al., 2019; Scholl et al., 2017; Takahashi et al., 2012; Varga et al., 2011; Wilson et al., 2016). My results present an important distinction
that could explaining these differences. Tectal neurons show clustered input tuning that is activity-dependent but stimulus-specific, due to mechanisms driving synaptic clustering only among inputs aligned with the cell’s spike tuning. Indeed, neurons using nonlinear integration to perform a computation would be expected to cluster inputs specific to that computation, and not other inputs. Such patterns may not be detectable in distributions of synaptic tuning to an arbitrary set of stimuli not aligned with an individual neuron’s spike tuning.

One unexpected finding was that there appeared to be some overlap in the encoding of the OFF response and the ON response in neurons, with some filopodia responding to both stimuli. While these filopodia showed a stimulus-driven response to both OFF and ON, they often appeared to show a preference to one stimulus over the other. The question raised by this finding is whether filopodia responding to both ON and OFF stimuli are driven by the activity of a single upstream axonal terminal. Alternatively, there is evidence that the filopodia of tectal neurons in *Xenopus* tadpoles can have more than one presynaptic contact (Li et al., 2011), but it is unknown what responsivity and tuning patterns of these multi-synaptic filopodia are. It is possible that a single filopodia having multiple synapses on it with at least one tuned to OFF and another tuned to ON. The result that the average amplitude of the calcium transient at the tips of filopodia for both the ON and OFF responses of filopodia tend to strengthen subsequent to spaced training to OFF suggests that the first scenario is more likely, and that there are presynapses on these filopodia that respond to both OFF and ON stimulus rather than multiple presynapses each only encoding OFF or ON. This is because if both responses were encoded by the same presynapses, strengthening of the ON response would occur as a by-product of spaced training driving the strengthening of the synapse through the OFF response. Results suggests
that the OFF and ON response pathways may not be completely separate, and that what is also being encoded at some synapses is also rapid shifts in contrast between light and dark.

The finding that both glutamatergic input and calcium response at filopodial tips are modified by spaced training opens the possibility that both presynaptic and postsynaptic plasticity is occurring in response to spaced training. Presynaptic plasticity can be measured directly through analyzing evoked iGluSnFR fluorescence over time, however post-synaptic plasticity is more challenging to directly confirm, since a change in the evoked calcium response could be driven by either a presynaptic or postsynaptic mechanisms. However, the finding that there are different proportions of filopodia where there is a training-induced change in synaptic weights, with a higher proportion of filopodia showing a change in the amplitude of the evoked calcium response then those showing a change in the amplitude of the evoked glutamate signal, suggests that some of the synaptic plasticity is driven by a post synaptic mechanism. Together, these results suggest both presynaptic and postsynaptic plasticity are occurring in these neurons in response to visual input.

These results demonstrate mechanisms directing experience-driven patterns of growth and pruning in developing brain neurons, and identify a set of rules that combine synaptic and spike activity signals to predict growth behavior throughout the dendritic arbor (Fig. 4-18). These rules allow neurons in developing brain circuits to strengthen or shift their spike tuning in response to strong sensory input. Results demonstrate how structured organization of synaptic distribution across the dendritic arbor arises during circuit formation by mechanisms promoting clustering of synapses tuned to the emerging somatic receptive field. Since the observed patterns of neuronal growth are directed by the salience of sensory stimuli to the tuning of each synapse on a developing neuron and the neuron’s spike tuning, my results reveal mechanism of
information-driven growth. In light of these findings, it opens the question about how enduring these experience induced functional and structural changes are. It has previously been found in the Haas lab that tectal neurons remain sensitive to spaced training induced potentiation up to tadpole stage 50. This suggests that there is a window where new environmental input might override training-induced potentiation. A modified version of these spaced training experiments with a follow-up imaging and activity recording session after an extended time with the tadpole in a natural environment would serve to shed light on the permanence of spaced training induced changes.

4.4 Summary of findings

Overall, these findings demonstrate that both glutamatergic input and the resultant relative levels of evoked calcium signals predict both growth and pruning patterns of dendritic filopodia for both a specific filopodia and its neighbors. Low activity in filopodia relative to adjacent shafts predicts high mobility and subtraction, while high activity in filopodia relative to adjacent shaft predicts both their structural stability and the addition of new filopodia in their vicinity, which respond to the trained stimulus. This is consistent with findings that stimulus-evoked glutamatergic input predicts a filopodia’s survival and the addition of nearby filopodia. Therefore, filopodia not synaptically linked to a strongly driven circuit are preferentially pruned, while connected filopodia are stabilized and promote new local filopodia additions that are both stable and responsive to the driven stimulus.

This research supports previous findings demonstrating that in developing neurons in the visual system, visual experience produces distinct patterns of structural changes in the dendritic
Importantly, since I have been able to record the complete repertoire of all stimulus-evoked calcium activity across all filopodia in parallel with recording structural changes I am able to make links between how experienced-induced structural and functional changes relate. These results suggest that as developing neurons become wired into stimulus-driven circuits responsive synapses act as epicenters for new growth while non-responsive synapses are eliminated. This offers an explanation of how the observed clustered topography of stimulus-evoked synaptic activity arises in mature animals (Iacaruso et al., 2017; Scholl et al., 2017; Wilson et al., 2016).

**Figure 4-18**

Synaptic input promotes patterned remodeling. Schematic of structural plasticity rules that drive clustering of similarly-tuned inputs. A neuron that lacks evoked somatic firing but has sparse input from a sensory circuit (left), can respond to strong stimulus training with which promotes clustered formation of new synapses responsive to the driven stimulus (center). Neurons that fire action potentials (spike tuned) to the driven stimulus exhibit high dendritic shaft calcium from
strong local synaptic activity and back-propagating action potentials, which promote subtraction of synapses and filopodia that are not connected to the driven circuit (right). This model demonstrates how brain neurons can shift and then refine their spike tuning.

**Figure 4-19**

*In vivo* spaced training experiment protocol. After the paralyzed tadpole is mounted in the stimulation chamber, the labeled neuron is located and a volume stack is taken from which the neuron’s structure is drawn by the operator. Volume stacks are then taken every 30 minutes to record changes in structure, during which the operator manually adjust the drawing to account for changes in dendritic arbor structure as well as drift. Subsequent to each drawing step, except that immediately prior to Spaced Training, the tadpoles are presented with Probing stimuli
consisting of 4 OFF stimuli and 4 ON stimuli presented in pseudorandom 8-12 second intervals. During this time, calcium activity is recorded across all filopodia, branch-points and the soma of the neuron using random access sampling at a rate of 6-10Hz.

4.5 Methods

4.5.1 Animal rearing conditions:

Albino *Xenopus laevis* tadpoles were reared in a room temperature container of 0.1x Steinberg’s solution (1x Steinberg’s in mM: 10 HEPES, 58NaCl, 0.67KCl, 0.34Ca(NO3)2, 0.83 MgSO4, pH 7.4). They were reared in a 12 hour day/night cycle. All experimental procedures and housing conditions were approved by the University of British Columbia Animal Care Committee and were in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

4.5.2 Expression of genetically-encoded fluorophores:

To record stimulus-evoked calcium activity, farnesylated (ie. membrane localized) jGCaMP7s was co-expressed with a farnesylated version of the red fluorophore mCyRFP1 (Laviv et al., 2016) using a plasmid containing a self-cleaving P2A (Kim et al., 2011) through employing single-cell electroporation (Haas et al., 2001). The red fluorophore mCyRFP1 served as a bright, photostable space-filler for tracking dendritic morphology. Single-cell electroporation parameters for this plasmid were 1.1s train duration, -40V, 1ms/pulse, and 200pulses/s. To image glutamatergic input in individual tectal neurons I expressed the fluorescence based high-sensitivity glutamate sensor iGluSnFR (SF-iGluSnFR-A184S) using SCE. Electroporation parameters were
0.4s train duration, -40V, 1ms/pulse, and 200 pulses/s. Neurons were screened for expression after 48 hours and imaged 72 hours post-electroporation.

4.5.3 Target neurons

For all experiments, we targeted type 13b pyramidal neurons in the dorsolateral tectum of Stage 48/49 tadpoles (Lazar, 1973). In this target region at this developmental stage, these neurons show robust visually evoked responses and are relatively stable morphologically, with motility rates that can be accurately captured by imaging at 30-minute intervals. Nevertheless, these neurons also robustly show both structural and functional plasticity with training.

4.5.4 In vivo imaging of single neuron activity:

Stage 48/49 Albino *Xenopus laevis* tadpoles were bathed for 5 minutes in 4mM pancuronium to temporarily paralyze them immediately prior to imaging. The tadpoles were then placed in a custom imaging chamber (Sakaki et al., 2020) that was perfused with oxygenated 0.1x Steinberg’s solution for the duration of the experiment. The structure and activity of the single labeled neurons were imaged at fast rates using a custom designed acousto-optic deflector (AOD)-based random access multiphoton microscope (Sakaki et al., 2020). Calcium dynamics throughout the dendritic arbors of individual neurons were detected as changes in jGCaMP7s fluorescence.

4.5.5 Visual stimulation and plasticity inducing protocol:

Visual stimuli were presented via a laser-projector (ShowWX+/PicoP, Microvision) placed near the eye contralateral to the imaged tectum. Where OFF stimuli were presented, the projector
(on at start of trial) was turned off for 50ms. Where ON stimuli were presented, the projector (off at start of trial) was turned on for 50ms. During each probing session, 4 OFF stimuli were shown, followed by 4 ON stimuli consisting of either ON or OFF flashes presented with pseudorandom inter-stimulus intervals ranging from 8-12 seconds. There was a slow shift between the ON background to the OFF background between each batch of 4 OFF and ON stimuli. Each probing session lasted approximately 10 minutes (Roughly 5 minutes where the optimal piezo motor route was plotted for each third of the neuron to be sampled and roughly 5 minutes where 3 batteries each composed of 4 OFF stimulus, a background transition and then 4 ON stimulus were presented as each third of the neuron was sampled independently) (Fig. 4-19). Morphological imaging, consisted of an initial image stack taken at the start of the experiment followed by 45 minute manual drawing of the complete neural structure through the RAMP-TPLSM user interface. Subsequent to this, there was a second image stack taken and over the next 10 minutes the drawing of the neuronal structure was manually updated to account for changes in arbor structure and drift. Prior to each presentation of a battery of probing stimulus another structural image stack was taken and the drawing was updated over the next 10 minutes (Fig. 4-19). Between periods where probing stimuli were presented and when structural image stacks were generated a ‘continuous probe’ OFF stimulus was given (50ms, OFF once per minute), which was previously demonstrated to have no potentiation effect (Dunfield & Haas, 2010). Spaced training consisted of three 5-minute bursts of high frequency (0.3Hz, 50 ms) OFF stimuli spaced by 5-minute periods of invariant light (Fig. 4-19).

Probing stimuli were presented in four epochs, 30 minutes prior to training, immediately post training at 30 minutes post training and at 60 minutes post training (Fig. 4-19). Structural recordings and drawing updates were taken immediately prior to each probing stimulus and also
an “update drawing lite” stack (roughly 3 minutes) was taken immediately prior to the start of spaced training, where no probing stimuli was presented. This was done to allow for the epoch between the structural recording prior to spaced training and immediately post-spaced training to be 30 minutes in length. Spaced training was performed between the first and second probing epoch, for a total stimulation period of 2 hours after the initial 45-minute drawing (Fig. 4-19).

4.5.6 Processing of calcium imaging data

The structural data along the functional recordings generated through the experiments performed on the RAMP-TPLSM were imported into a custom designed morphometrics software suite, Dynamo (Coleman et al., n.d.). Using Dynamo, changes in the arbor structure over time were tracked and motility at filopodia was labeled as additions, subtractions, extensions and retractions. The raw calcium signal was then converted to $\Delta F/F_0$ using the specifications in previously published research (Jia et al., 2011). An Okada filter was applied to the traces to reduce shot noise on noisy activity data by flattening out samples that are abnormally higher or lower than the samples around them (Okada et al., 2016). Next, the $\Delta F/F_0$ signal was smoothed using an in-house designed non-negative deconvolution (‘NND’) approach used in prior research utilizing this model (Podgorski & Haas, 2013).

To determine if a filopodia was responsive to a stimulus, the raw fluorescence intensity for both the filopodia tip and base were converted to $\Delta F/F_0$, filtered and smoothed. Next, noise levels for both the tip and the base were calculated by measuring the median standard deviation at times when no stimuli are presented. A filopodia was classified as responding to a stimulus if, after the stimulus was presented, the $\Delta F/F_0$ at the tip was greater than the base by at least the noise level of that trace (the standard deviation of the $\Delta F/F_0$ for 3 seconds pre-stimulus). This was done to avoid
false-positives from back-propagating action potentials (bAP), where the filopodium is inactive but calcium levels increase across the arbor, decreasing in intensity as it travels distally. For analysis of filopodial tip transients, the smoothed ΔF/F0 traces at each timepoint were processed through an in-house designed event detector and then manually reviewed for accuracy. A filopodia was defined as “Potentiated” for a specific epoch if the average amplitude of the stimulus-evoked events at that epoch was at least 50% above the average amplitude of stimulus-evoked events during pre-training, and to have “Weakened” if average amplitude of the stimulus-evoked events at that epoch was at least 50% below pre-training.

4.5.7 Grouping of neurons by activity and plasticity

For all experiments, neurons were classified as baseline OFF responsive or not based on somatic and, when available, axonal calcium recordings were action potentials were detected in response to at least 50% of presented off stimulus using a matched-filter (MF) based event detector described and validated in these Xenopus tectal neurons previously (Sakaki et al., 2018). Neurons were grouped based on both their input and output potentiation. For grouping based on action potential output, “Long-term potentiation” neurons were responsive before, during and after spaced training to OFF and showed at least a 10% increase in evoked amplitude over two consecutive timepoints. “Short-term potentiation” neurons were responsive before and during spaced training to OFF and showed at least a 10% increase in evoked amplitude during spaced training. Neurons that were grouped as “Become responsive” were not responsive to OFF at baseline but were during training. “Stable” neurons showed baseline responsiveness to OFF but did not potentiate during or after spaced training to OFF. “Non-responsive” neurons were neurons that were not responsive to OFF stimulus before or during training. Neurons were divided by
whether they showed dendritic potentiation or not based on whether they showed a significant increase in average evoked filopodia tip amplitude during or post-training (peak ΔF/F₀>0, p<0.05, Dunnets test).

4.5.8 Spatial clustering

To measure spatial clustering of various properties I computed the nearest neighbor distance (NND) for that property. For example, the distance between a filopodia to the nearest filopodium that responded to the OFF stimulus would be that filopodia’s OFF NND. We excluded filopodia at the tips of branches ('terminal filopodia') from our clustering analyses; terminal filopodia show different patterns of motility than interstitial filopodia (Hossain et al., 2012), and are inherently clustered by virtue of being at branch tips. To measure changes in clustering across timepoints, I compared the average observed NNDs at those timepoints. To validate significant results I also compared the observed NND to the expected under the null (non-clustered) distribution, obtained by Monte Carlo sampling. The null distribution was calculated by uniform randomly reassigning the observed number of addition sites along the length of the dendritic arbor. These reassignments were performed 500 times for each neuron to generate the null distribution for each quantity.

4.5.9 Statistics

Statistical tests are reported alongside p-values. Python code for Monte Carlo simulations and statistical tests are available upon request.
Chapter 5: Manuscript 2- An algorithm based on a Cable-Nernst Planck model predicting synaptic activity throughout the dendritic arbor with micron specificity

5.1 Preface: Designing and validating a model of synaptic driven calcium flux in an in vivo model system

Through my fast-imaging experiments using the RAMP-TPLSM to record tectal neurons in Xenopus tadpoles receiving visual stimulus, I had achieved fast imaging of neurons in vivo, being capable of recording both stimulus-evoked and spontaneous calcium activity across the complete dendritic arbor. This technical capability has unlocked new avenues of inquiry, analyzing how developing neurons inputs and structure are shaped by experience and ultimately how they become tuned to particular stimuli and eventually wired into mature circuits. However, it also led to a need to develop models capable of distinguishing the range potential sources of calcium fluxes across the cell membrane in a neuron, particularly with an emphasis on trying to discriminate synaptic inputs. To accomplish this I collaborated with a leading mathematician in the area of neural activity modelling, Professor Claire Guerrier. Together, along with a computer programmer Nicolas Galtier, we attempted to generate a computer model of calcium fluxes from multiple sources informed by experimental findings in these tectal neurons and to then to compare the modelled data with real-world experimental data. The modelled data was able to reproduce experimental findings and ultimately it provides a tool capable of predicting the locations of synaptic activity in these neurons.

5.2 Summary
Recent technological advances have enabled the recording of neurons in intact circuits with a high spatial and temporal resolution, creating the need for modeling with the same precision. In particular, the development of ultra-fast two-photon microscopy combined with fluorescence-based genetically-encoded Ca\(^{2+}\)-indicators allows capture of full-dendritic arbor and somatic responses associated with synaptic input and action potential output. The complexity of dendritic arbor structures and distributed patterns of activity over time results in the generation of incredibly rich 4D datasets that are challenging to analyze (Sakaki et al., 2020). Interpreting neural activity from fluorescence-based Ca\(^{2+}\) biosensors is challenging due to non-linear interactions between several factors influencing intracellular calcium ion concentration and its binding to sensors, including the ionic dynamics driven by diffusion, electrical gradients and voltage-gated conductances. To investigate those dynamics, we designed a model based on a Cable-like equation coupled to the Nernst-Planck equations for ionic fluxes in electrolytes. We employ this model to simulate signal propagation and ionic electrodiffusion across a dendritic arbor. Using these simulation results, we then designed an algorithm to detect synapses from Ca\(^{2+}\) imaging datasets. We finally apply this algorithm to experimental \(\text{Ca}^{2+}\)-indicator datasets from neurons expressing jGCaMP7s (Dana et al., 2019), using full-dendritic arbor sampling in vivo in the *Xenopus laevis* optic tectum using fast random-access two-photon microscopy. Our model reproduces the dynamics of visual stimulus-evoked jGCaMP7s-mediated calcium signals observed experimentally, and the resulting algorithm allows prediction of the location of synapses across the dendritic arbor. Our study provides a way to predict synaptic activity and location on dendritic arbors, from fluorescence data in the full dendritic arbor of a neuron recorded in the intact and awake developing vertebrate brain.
5.3 Introduction

A leading question in neuroscience is how neurons process synaptic inputs and generate an output encoded in action potential activity. In particular, it remains unanswered how the geometry of the dendritic arbor and distribution of the synapses contribute to the integration of synaptic information and the computation of the spike output. The historical model that dendrites serve to merely connect neurons and to passively convey information is currently challenged by recent findings demonstrating that the shape of the dendritic arbor, and by extension, the location of the synaptic inputs is key for non-linear signal integration (Gonzalez et al., 2022; Lavzin et al., 2012; Redmond & Ghosh, 2005). Indeed, dendritic patch-clamp electrophysiology and imaging of genetically encoded fluorescent Ca$^{2+}$-indicators have made recording activity within the dendritic arbor increasingly feasible, highlighting its involvement in information processing at a cellular level (Basak & Narayanan, 2018; London & Häusser, 2005). As a result, defining the role of the dendritic arbor and synaptic topography in information processing and encoding has become a leading area of research in developmental neuroscience. This field has taken a sizable leap forward by recent advances in ultra-fast multi-photon calcium imaging that provide unprecedented spatial and temporal resolutions of comprehensive dendritic calcium dynamics in intact and awake animals, but which also present novel challenges for their analysis (Sakaki et al., 2020).

Relating the observed calcium dynamics, generated from full-dendritic arbor imaging of neurons expressing fluorescence-based calcium sensors, to underlying neural activity remains challenging. The regulation of intracellular calcium concentration by activity is complex, due to multiple sources of calcium and their nonlinear interactions. Calcium ions can enter neurons through synaptic and extrasynaptic glutamatergic receptors, and voltage-gated calcium channels,
and can be released from intracellular endoplasmic reticulum stores. For studies focusing on tracking synaptic activity, there is a need for modeling to differentiate multiple calcium sources in order to determine the location and amplitude of synaptic responses. Such modeling requires understanding how voltage propagates throughout the dendritic arbor and its influence on ionic concentrations, especially in small neuronal compartments such as filopodia and dendritic spines (Holcman & Yuste, 2015; Savtchenko et al., 2017).

Numerous models have been generated to characterize voltage dynamics in neurons. The standard approach consists of using Cable theory with Hodgkin-Huxley formalism (Bower & Beeman, 1998; Carnevale & Hines, 2006; Qian & Sejnowski, 1989), or integrate-and-fire type modeling (Brette & Gerstner, 2005; Harkin et al., 2022; Keener et al., 1981). Most of these models are geared toward neuronal network simulation, and do not include ionic dynamics. Other models are focused on ionic dynamics, and are based on the Nernst-Planck equation describing ionic fluxes, coupled to the Poisson equation, or to an electroneutral model (Lopreore et al., 2008; Lu et al., 2010; Mori et al., 2008; Pods et al., 2013; Sætra et al., 2020; Solbrå et al., 2018; Xylouris et al., 2010). The high non-linearity of the Poisson-Nernst Planck system of equations, and the presence of a thin boundary layer at the membrane renders such simulation and analysis a daunting task, especially when taking the complex geometries of dendritic arbors into consideration (Cartailler et al., 2017; Savtchenko et al., 2017).

Here, our goal is to realize fast simulations of voltage and ionic dynamics in detailed dendritic arbor geometries. To achieve this, we developed a model and simulations for voltage propagation and ionic electrodiffusion in the dendritic arbor. The model is based on a coupling
between the Nernst-Planck equations to represent ionic fluxes due to the electrodiffusion of ions (Kirby, 2010), and a Cable-like equation representing voltage dynamics. We demonstrate that under specific assumptions, we can reduce the total ionic flux to a simple resistive flux, and hence decouple the equations, while accurately keeping track of calcium dynamics. This decoupling simplifies the program and enables faster simulations. Simulations of the simplified model are performed using the Sinaps Python library the authors developed previously (Galtier & Guerrier, 2022).

To validate the model, we compared simulation results with data from in vivo two-photon calcium imaging experiments, using neurons expressing the genetically encoded fluorescent protein jGCaMP7s. We observed a substantial discrepancy between the temporal scales of the action potential and jGCaMP7s fluorescence dynamics: from the duration of the action potential, ranging from sub-millisecond to several milliseconds, to the entry of calcium through voltage-gated channels that takes several milliseconds and persists for tens of milliseconds, and finally to the fluorescence response of calcium sensors as observed in the experimental data, that last for multiple seconds. Importantly, we found that the locations of fluorescence dynamics across the arbor proximate to synapses are different from the dynamics distant from a synapse. This allows for the discrimination and localization of potential synaptic activity from fluorescence-based calcium data.

In this paper, we first describe our model coupling the Nernst-Planck equations and a Cable-like equation in the full dendritic tree, and show that this system can be decoupled, to speed-up simulations. Next, using the decoupled system, we simulated calcium and jGCaMP7s dynamics in
a full neuronal geometry. From these simulated results, we infer that the calcium dynamics as reported by the fluorescence changes in genetically encoded calcium sensors differ depending on the distance of the sampled point from an active synapse. We then propose an algorithm to detect possible synaptic activity in fluorescent datasets and test it on experimental measurements.

5.4 Methods

5.4.1 Experimental protocol

Animal rearing conditions: Albino *Xenopus laevis* tadpoles were reared in a room temperature container of 0.1x Steinberg’s solution (1x Steinberg’s in mM: 10 HEPES, 58NaCl, 0.67KCl, 0.34Ca(NO3)2, 0.83 MgSO4, pH 7.4). They were reared in a 12 hour day/night cycle. All experimental procedures and housing conditions were approved by the University of British Columbia Animal Care Committee and were in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

Expression of genetically-encoded fluorophores: Single-cell electroporation (Haas et al., 2001) was employed to express EGFP in tectal neurons (Dana et al., 2019; Sakaki et al., 2020). Electroporation parameters were 300ms train duration, -40V, 1ms/pulse, and 200pulses/s. The tadpoles were screened for expression of EGFP in single neurons after 48 hours, and imaged 72 hours post-electroporation. To record stimulus-evoked calcium activity, farnesylated (ie. membrane localized) jGCaMP7s was co-expressed with a farnesylated version of the red fluorophore mCyRFP1 (Laviv et al., 2016) using a plasmid containing a self-cleaving P2A (Kim
et al., 2011). The red fluorophore mCyRFP1 served as a bright, photostable space-filler for tracking dendritic morphology. Single-cell electroporation parameters for this plasmid were 1.1s train duration, -40V, 1ms/pulse, and 200 pulses/s.

**In vivo imaging of single neuron activity:** Stage 48/49 Albino *Xenopus laevis* tadpoles were bathed for 5 minutes in 4mM pancuronium to temporarily paralyze them immediately prior to imaging. The tadpoles were then placed in a custom imaging chamber (Sakaki et al., 2020) that was perfused with oxygenated 0.1x Steinberg’s solution for the duration of the experiment. The structure and activity of the single labeled neurons were imaged at fast rates using a custom designed acousto-optic deflector (AOD)-based random access multiphoton microscope (Sakaki et al., 2020). Calcium dynamics throughout the dendritic arbors of individual brain neurons were detected as changes in jGCaMP7s fluorescence. Branch radii were derived from a 3D image stack of the volume containing the complete neuron prior to the recording of calcium data.

**Visual stimulation protocol:** Visual stimuli were presented to the eye contralateral to the imaged tectum using a projector. Stimuli were composed of full-field brief (50 ms) flashes of OFF or ON stimuli, presented as a series of 4 pseudo-randomly spaced OFF stimulus (presented between 8-12 seconds apart) on an ON background, followed by a transition shift from an ON background to an OFF background and then 4 ON stimulus on an OFF background.

**Estimation of proportion of tuned vs untuned synapses in a dendritic arbor:** The value was generated from a dataset of 6 jGCaMP7s expressing *Xenopus* tectal neurons receiving OFF visual stimuli that produce stimulus-evoked action potential outputs. The calcium activity of all terminal dendritic branches was recorded, and the ratio of active branches was estimated by taking the
proportion of branches with localized stimulus-driven branch-tip activity compared to non-responsive branches.

5.4.2 Mathematical modeling

**Nernst-Planck equation**: Our model for ionic electrodiffusion and voltage dynamics in the dendritic arbor couples the Nernst-Planck equation with an electrical model describing membrane voltage. The Nernst-Planck equation describes the motion of ionic species in a fluid driven by both thermal diffusion, and electrostatic forces. This equation is built from the first principle of mechanics, stating the conservation of ions (Nernst, 1888):

\[
\frac{\partial c_i}{\partial t} = - \nabla \cdot j_i \quad \text{for all ions } i,
\]

where \( c_i \) is the concentration of ion \( i \) in \([\text{mol.m}^{-3}]\). The flux of charge \( j_i \) in \([\text{mol.s}^{-1}.\text{m}^{-2}]\) sums the diffusion flux of ionic species derived from Fick’s law \( j_{\text{diff}}^i = -D_i \nabla c_i \), and an electric flux representing the motion of ions due to the electric field: \( j_{\text{elec}}^i = -D_i \frac{z_i e c_i}{k_b T} \nabla V \) (Kirby, 2010; Nernst, 1888). In this equation, \( V \) represents the voltage, and \( \frac{D_i z_i e c_i}{k_b T} \) the Einstein relation for the mobility of ion species: \( D_i \) is the diffusion coefficient of species \( i \), \( z_i \) the valence, \( k_b T \) the thermal energy and \( e \) is the electric charge (Kirby, 2010). We get

\[
j_i = j_{\text{diff}}^i + j_{\text{elec}}^i = -D_i \nabla c_i - D_i \frac{z_i e c_i}{k_b T} \nabla V. \quad \text{#(2)}
\]
A dendritic arbor is a complex tree-like structure where different compartments have various radii (Fig. 5-1A). We model this neuronal geometry by setting each dendritic segment as a cylinder with a specific radius (Fig. 5-1B). We consider the integrated flux along one branch of the tree of radius $a$, $J_i = \pi a^2 j_i$ in [mol.s$^{-1}$], which gives a one-dimensional ionic flux in space (Fig. 5-1B). To add the contribution of ionic fluxes coming from membrane channels, we consider $j^{m,i}$, the flow per unit surface of species $i$ coming from the channels (in [mol.s$^{-1}$.m$^{-2}$]). Considering the radius $a$ of the cylindrical branch, the integrated flux over the membrane becomes $f^{m,i} = 2 \pi a j^{m,i}$ (Fig. 5-1B).

Finally, eq. 1 becomes:

$$\pi a^2 \frac{\partial c_i}{\partial t} = - \nabla \cdot J_i + f^{m,i} \tag{4}$$

for all ions $i$.

**Chemical reactions:** We consider general chemical reactions in the cytosol, taking the form:

$$\sum_{i=1}^{n} \alpha_i A_i \Leftrightarrow \sum_{i=1}^{m} \beta_i B_i \tag{5}$$

between ionic species $A_i$, $i = 1, ..., n$ and $B_i$, $i = 1, .., m$, with forward and backward reaction rates $k_f$ and $k_b$ respectively. The forward and backward reaction speeds are respectively:

$$v^f(x,t) = k_f \prod_{i=1}^{n} \left( c^A_i(x,t) \right)^{\alpha_i}$$

$$v^b(x,t) = k_b \prod_{i=1}^{n} \left( c^B_i(x,t) \right)^{\beta_i}$$

where $c^K$ represents the concentration of species $K$. Hence, the variation of concentration of each species, due to the reaction at position $x$ and at time $t$ is:

$$\gamma_{A_i}(x, t) = \alpha_i (v^b(x,t) - v^f(x,t))$$
for the reactants $A_i$, and

$$\gamma_{B_i}(x, t) = \beta_i(v^f(x, t) - v(b, x, t))$$

for the products $B_i$. The final equation for the variation of concentration of each ionic species is given by:

$$\pi a^2 \frac{\partial c_i}{\partial t} = -\nabla \cdot J_i + J^{m,i} + \pi a^2 \gamma_i \#(6) \text{ for all ions } i.$$

**Voltage dynamics:** The electric flux $j_{\text{lec}}^i$ of species $i$ in the Nernst-Planck equation (eq. 2), requires the voltage profile along the branches. To build an equation for the voltage, we assume that the neuronal membrane behaves as a capacitance, i.e. the total charge density $q$ (in $[\text{C.m}^{-2}]$) at the membrane is proportional to the voltage difference $V$ (in $[\text{V}]$) across the membrane, with proportionality coefficient $c_{\text{cap}}$ (in $[\text{F.m}^{-2}]$):

$$q = c_{\text{cap}} V \#(7).$$

Integrating over the membrane, we get the lineic charge $Q = 2\pi a q$, in $[\text{C.m}^{-1}]$. Using that the center of the branch is electroneutral (Stinchcombe et al., 2016), and hence the charges are all located at the membrane, the charge $Q$ at the membrane is equal to the integrated charge over the section of the branch: $Q = \pi a^2 \sum_{i \text{ ions}} c_i z_i N_a e$ where $N_a$ is the Avogadro number. Deriving eq. (7) over time, we obtain using the expression for $Q$:

$$c_{\text{cap}} \frac{\partial V}{\partial t} = \frac{a}{2} \sum_{i \text{ ions}} \frac{\partial c_i}{\partial t} z_i N_a e. \#(8)$$

The system of equations given by eq. (1, 2) and (6) for all ions, coupled to eq. (8) defines our model for voltage and ionic electrodiffusion in dendritic branches. The equations defining the channels dynamics $f^{m,i}$ are described below.
Membrane channels dynamics: To model the influx of ions through voltage-gated channels, we use a simplified Hodgkin-Huxley model for the sodium, potassium and leak channels (Hodgkin & Huxley, 1952), to which we add a Hodgkin-Huxley type channel for calcium current (Guerrier & Holcman, 2017). The classical Hodgkin-Huxley dynamics for the sodium, potassium and leak currents is reduced to a 2-dimensional system, using the approximation $h = (0.89 - 1.1n)$, and $m = m_{\text{inf}}$ (Izhikevich, 2006):

\[
\frac{dn}{dt} = 0.1 \alpha_n (1 - n) - \beta_n n
\]

\[
I_{Na} = g_{Na} m_n^3 h (V - E_{Na})
\]

\[
I_K = g_K n^4 (V - E_K)
\]

\[
I_L = g_L (V - E_L)
\]

\[
m_{\text{inf}} = \frac{\alpha_m}{\alpha_m + \beta_m}
\]

and for $k = n,m$:

\[
\alpha_k = \frac{1}{\tau_k} \frac{\theta_k - V}{\exp\left(\frac{\theta_k - V}{\sigma_k}\right) - 1}
\]

\[
\beta_k = \eta_k \exp\left(\frac{-V}{\sigma_k}\right) \text{ for } k = n, m.
\]

The membrane resting potential of the cell is 0 mV. The calcium current dynamics is given by (Guerrier & Holcman, 2017):

\[
I_{Ca} = g_{Ca} p^3 l (V - E_{Ca})
\]
and for $\kappa = p,l$:

$$\frac{d\kappa}{dt} = \frac{1}{\tau_\kappa} \left( \frac{1}{1 - \exp(\theta - V)} - \kappa \right).$$

The sodium, potassium and calcium currents are ion specific, and thus can be easily converted into ionic fluxes through the formula

$$J^{m,i} = \frac{I_i}{N_z e i}.$$ To maintain coherence within the Hodgkin-Huxley model, we consider that the leak current is only driven by chloride.

We included N-methyl D-aspartate (NMDA) receptors in our simulations, to mimic excitatory postsynaptic potentials. The mean approximation for the current entering the domain is (Koch, 2004):

$$I_N(t) = g_N e^{-t/\tau_{\text{NMDA}}} - \frac{1}{1 + 0.33[Mg^{2+}]e^{-\Delta E_N}} (V - E_N),$$

where we use the NMDA receptors conductance $g_N = 0.2$ nS and Nernst potential $E_N = 0$.

To estimate the total amount of calcium ions entering through the NMDA receptors, we use that the fraction of the current carried by calcium ions is 15%. All parameters are given in Table 1.

**Decoupled model:** We decoupled the system of equations (2)-(6) and (8), using two assumptions: the first assumption is that the total diffusive flux is negligible compared to the total electric flux $\sum_i z_i J_{\text{diff}}^i \ll \sum_i z_i J_{\text{elec}}^i$. The second assumption is that the electric conductivity of the cytoplasm $\sigma = \sum_i N_{\text{ion}}^i \frac{D_{\text{ion}}^i \theta_i e^e c_i}{k_b T}$ is constant (in [S.m$^{-1}$]). Using these two assumptions combined with eq. (1)-(2), we recover that the electric current along one branch is proportional to the gradient of the voltage:

$$I_R = N_e \sum_{i, \text{ions}} z_i J_i = \pi \alpha^2 \sigma \nabla V.$$ We finally get the standard cable equation for the voltage:
\[ c_{\text{cap}} \frac{\partial V}{\partial t} = I_m + \frac{a}{2} \sigma \frac{\partial^2 V}{\partial x^2} \tag{9} \]

where \( 2\pi a l_m = N_a e \sum_{i} z_i J_{im} \).

Hence, the strategy of the decoupled model is to first compute the voltage using eq. (9), and then plug this dynamics into the Nernst-Planck equations eq.(1)-(2), and solve the ordinary differential equation for the ionic species we are interested in. Simulations of this simplified model are realized with the Sinaps python library the authors developed previously (Galtier & Guerrier, 2022).

### 5.4.3 Data analysis

**Data filtering procedures - data normalization:** We observe a substantial variability in the fluorescence data between different nodes before the visual stimulation, during which the neuron is not receiving stimulus-evoked synaptic inputs. To explain this variability, we note that the fluorescence data reflects the number of photons received in a fixed period of time, and hence is representative of the number of jGCaMP7s-Ca present at the focal point of the laser position. For a given concentration \( c_0 \) of jGCaMP7s-Ca, in a dendrite of radius \( a \), the number of observed photon will be proportional to \( \pi a^2 c_0 \). Hence, the fluorescence can vary with the arbor radii.

This explains why the soma in the experimental data is very bright compared to the rest of the arbor, and why small filopodia are typically dim. To estimate the concentration of jGCaMP7s-Ca, we normalized each trace so that the mean fluorescences observed during the time before the first visual stimuli are the same. The fluorescence data were normalized only at this stage, to maintain a similar level of noise in each trace.
Data filtering procedures - denoising: To remove noise in the fluorescence data, we applied a rolling window filter to the raw data. We used a gaussian window, defined as \( w(n) = e^{-n^2/2\sigma^2} \) with the window size \( n = 50 \) data points and the standard deviation parameter \( \sigma = 10 \).

Synapse localization algorithm: To localize synaptic activity, we designed an algorithm based on the rising dynamics of jGCaMP7s-Ca. We first applied our filtering procedure to generate a smooth curve representing the data as described above. At each recorded spatial location, we obtained an estimate of the rising slope of the time-dependent signal by taking the derivative in time at 60 ms following the visual stimuli. We then detected local maxima of the rising slope in space, which gave us the potential active synapses locations. A local maxima is defined as a point at the max over its five neighboring points.

5.5 Results

Simulation of the coupled model: We implemented the coupled model given by eq. (2)-(6) and (8) (Method section, Fig. 5-2), in a simple branch attached to a soma. At the soma, we set up a Hodgkin-Huxley dynamics with sodium, potassium, and leak, as well as voltage-gated calcium channels (Method section). The soma radius and length are 2.5 and 5 \( \mu \)m respectively, and the branch radius and length are 1 and 50 \( \mu \)m. The ion flow throughout the branch is passive. We applied a current of amplitude 7 pA lasting 0.25 ms at the soma, to trigger an action potential. We plotted the potential and calcium dynamics, as well as the electrical conductivity, and the calcium diffusive and electric currents (Fig. 5-2, solid lines). We observe, as expected, that the action potential propagates very quickly within the branch (Fig. 5-2A, solid lines). We also observe a rise
in calcium concentration due to the entry of calcium through voltage-gated calcium channels at the soma. This calcium propagates inside the branch due to the diffusive and electric fluxes (Fig. 5-2B, solid lines). To understand the contribution of both fluxes to calcium dynamics, we plotted $j_{elec}^{Ca}$ and $j_{diff}^{Ca}$ in the soma and at one position inside the branch (Fig. 5-2C). The maximum of the electric calcium flux is around $1.5 \times 10^{-3} \mu\text{mol.}\mu\text{m}^{-2}\cdot\text{ms}^{-1}$ in both the soma and the branch, whereas the maximum of the diffusive flux is around $5.7 \times 10^{-5} \mu\text{mol.}\mu\text{m}^{-2}\cdot\text{ms}^{-1}$ in the soma and drops to $1.7 \times 10^{-7} \mu\text{mol.}\mu\text{m}^{-2}\cdot\text{ms}^{-1}$ in the branch. We also observe that the electric flux is on a faster time scale compared to the diffusive fluxes. These properties are maintained for sodium, potassium and chloride ions. This shows that the ionic dynamics in this framework are driven by electric fluxes, and support the hypothesis used in the decoupled model: $\sum_i z_i j_{diff}^i \ll \sum_i z_i j_{elec}^i$.

We also plotted the variations of the electrical conductivity of the cytosol $\sigma = \sum_{ions} \frac{D_i \rho_i \sigma_i}{k_B T}$ in Fig. 5-2D. We observed a maximal variation of up to 0.05 % in $\sigma$, which is coherent with the second assumption of the decoupled model, that $\sigma$ is constant.

**Figure 5-1**

A one-dimensional model of a three-dimensional dendritic arbor. (A) Z-stack of a Xenopus tectal neuron expressing EGFP, imaged while the animal was awake. Inset image is a 4.0x zoom of a
section of dendrite with progressive thinning modeled in the panel to the right. (B) Schematic representation of our modeling approach for two nodes of a dendritic branch analogous to the inset in A. The entire dendritic arbor is represented as a tree structure containing many nodes, where each branch of the tree is represented by multiple three-dimensional cylinders. Integrating over the cylindrical geometry, the model is reduced to a one-dimensional tree structure.

**The coupled vs decoupled model:** The coupled model can be simplified by decoupling the two equations which results in faster simulation. Our strategy is to first compute the voltage dynamic inside the neurons (eq. 9), and plug this voltage into the calcium concentration equation to recover the dynamics of calcium (eq. 1, see Method section for further details). We show in Fig. 5-2 the difference in calcium and voltage dynamics in the coupled model (solid lines) versus decoupled model (dashed lines). We observe no differences between the two curves in voltage dynamics, and a small difference of less than 4% in calcium dynamics. The differences in sodium and potassium dynamics are less than 1%. To simulate voltage dynamics and ionic electrodiffusion in a full dendritic arbor geometry, we will utilize the decoupled model.
Figure 5-2

Comparison between the coupled and the decoupled model: Simulation of ionic dynamics in an active soma, connected to a passive branch. An applied current triggers an AP in the soma that propagates to the branch. Comparison between the coupled (solid lines) and the decoupled (dashed lines) models, at three different sites: the soma, and the base and tip of the branch. Time dynamics of voltage (all lines overlap) (A), calcium concentration (B) and electrical conductivity $\sigma$ (C) at the soma and along the branch. (D) Time dynamics of the currents $J_{elec}^{Ca} = \pi a^2 j_{elec}^{Ca}$ (blue and red) and $J_{diff}^{Ca} = \pi a^2 j_{diff}^{Ca}$ (purple and pink) at the soma, and in the dendritic branch 5 $\mu$m from the soma.

In both the coupled and decoupled models, we keep track of all the ionic species, using the standard ionic concentrations as has been previously described (Alberts et al., 2002). Due to the absence of sodium, potassium, and calcium reuptake, which all take place at a slower time scale, the concentrations of the different ions do not return to their resting values after an action potential. As a consequence, the change in the electric conductivity $\sigma$ past 20 ms is due to an imprecision
of our model at long time scales. To fully describe the variation of ionic concentrations in the cytosol in a longer time scale, it is necessary to add to this framework a model for the Na\textsuperscript{+}/K\textsuperscript{+} ATPase exchanger (Øyehaug et al., 2012), as well as for calcium extrusion, such as NCX or PMCA.

**Simulation of calcium and jGCaMP7s dynamics in a dendritic arbor:** Our goal is to realize numerical simulations recapitulating the fluorescence recordings obtained in the dendritic arbor of brain neurons recorded from *Xenopus laevis* tadpoles *in vivo*. Awake tadpoles expressing a membrane-localized jGCaMP7s in individual brain neurons were immobilized and visual stimuli were presented while full-neuron morphology and fast imaging of calcium dynamics were recorded using a random-access multiphoton microscope (Sakaki et al., 2020).

**Figure 5-3**
Simulation of voltage, calcium and jGCaMP7s-Ca dynamics in a dendritic arbor. (A) Left: Tree-like representation of a simulated neuron, derived from the morphology of a real neuron expressing
jGCaMP7s. Middle and Right: Comparison between the time scales of voltage, calcium and jGCaMP7s-Ca concentration dynamics in simulated dataset, with the position of recorded activity indicated by the colors on the y-axis corresponding to color labels on the neuronal structure. The blue stars on the y-axis in the jGCaMP7s-Ca field plot represent synapses locations. (B) Time dynamics of normalized voltage, calcium and jGCaMP7s-Ca concentration. (C) Magnification of the traces in B.

We imported the full dendritic geometry of a neuron using our Python library, Sinaps (Fig. 5-3A). Our dataset includes the radii, ranging from 1µm (across the dendrites) to 8.5 µm (across the soma), with a mean of 2.8 ± 2.5 µm. The dendritic geometry is represented as a tree structure, composed of nodes every 2 µm, which gives a total of 268 nodes. The terminal portion of a branch (i.e. not connected to a new branch) is called a leaf. We then modeled the influx of ions into the dendritic tree through different channels: to represent active channels, we implemented Hodgkin-Huxley type channels with potassium, sodium, calcium and leak. We set Hodgkin-Huxley channels to be distributed everywhere on the surface of the dendritic arbor. At the soma, we set Hodgkin-Huxley channels with a conductance multiplied by 3. This was informed by previous modeling replicating experimental results that indicate that the soma has a higher density of voltage gated ionic channels (Bono & Clopath, 2017). To mimic synaptic activity, we added NMDA receptors to several nodes (see complete description in Method) (Li et al., 2011; Wu et al., 1996). To determine which node will be given a synapse, we used the results on synaptic repartition given in (Li et al., 2011). First, Li et al. (2011) observed that 93% of terminal dendritic branches are receiving synaptic contact. To account for this proportion, we randomly chose 22 leaves over the 24 of our tree and added a synapse at those leaves. Second, the average synapse density reported
in (Li et al., 2011) is 0.43 synapses.µm\(^{-1}\). The dendritic arbor we are considering is 534 µm long, which corresponds to approximately 229 synapses. Considering that we already set 22 synapses, we randomly distribute the 207 remaining synapses along the tree. Finally, we account for the fact that even in neurons that produce action potentials in response to a stimulus, only a subset of synapses on that neuron will be tuned to be responsive to that particular stimulus by setting, for each synapse, a probability of being tuned to a stimulus of 0.24 (see Methods).

Calcium dynamics inside the cytosol are then coupled to the jGCaMP7s dynamics through the chemical reaction:

\[ \text{Ca}^{2+} + \text{jGCaMP7s} \leftrightarrow \text{jGCaMP7s} - \text{Ca} \quad \#(9) \]

with association rate \( k_f = 21.5 \text{ mM}^{-1}.\text{ms}^{-1} \) and dissociation rate \( k_b = 0.00286 \text{ ms}^{-1} \) (Dana et al., 2019). We model calcium extrusion from the cell using a simple reaction \( \text{Ca}^- > \emptyset \) with reaction rate \( k_{ex} = 0.03 \text{ ms}^{-1} \). The initial calcium concentration in the cytosol is \( c^{Ca} = 10^{-4} \text{ mM} \), and the jGCaMP7s concentration is \( c^{jGCaMP7s} = 5 \cdot 10^{-3} \text{ mM} \). We performed simulations for different values of the jGCaMP7s concentration, ranging from \( 10^{-3} \text{ mM} \), to \( 10^{-2} \text{ mM} \), and observed no qualitative changes in the results. Simulations with the jCGaMP7s initial concentration \( c^{jGCaMP7s} \) around \( 10^{-3} \text{ mM} \) and below resulted in buffer saturation throughout the arbor, which is not consistent with experimental results. In simulations with \( c^{jGCaMP7s} \) above \( 10^{-2} \text{ mM} \), we observe that jGCaMP7s is in excess, which likewise does not match with experimental observations. As the jGCaMP7s and jGCaMP7s-Ca employed here are membrane-bound (see Methods), we assume that their diffusion in the membrane is negligible. We represent the arrival of an input coming from the visual pathway via retinal ganglion cells (RGC), as a train of 6 synaptic inputs at 40 Hz (Demas et al., 2012; Honda
et al., 2011). Synaptic inputs are modeled through the opening of NMDA receptors at synapses.

We perform numerical simulations using the Python library Sinaps (Galtier & Guerrier, 2022), Fig. 5-3. From this, we observe a time scale difference between the action potential dynamics, that lasts for a few ms, to the calcium concentration dynamics, lasting a few hundred milliseconds, to the fluorescence calcium sensor dynamics lasting several seconds (Fig. 5-3B-C). Additionally, we observe that the slower time scale of jGCaMP7s-Ca dynamics ‘filters’ voltage and calcium signals, making it more difficult to infer calcium and voltage dynamics from jGCaMP7s-Ca data.

**jGCaMP7s-Ca rise dynamics are faster when proximate to synaptic activity:** The simulations of jGCaMP7s-Ca dynamics in the full dendritic arbor reveals the filtering effect of the change in time scale (Fig. 5-3B). Hence, the detailed dynamics of calcium entry is not easily observable in fluorescence data (Fig. 5-4A). We also observe that the region close to the soma has the highest stimulus-evoked fluorescence amplitude, which is due to the large influx of calcium ions at the soma. This shows that the maximum intensity of fluorescence over the tree is not a good marker of synaptic activity. We also observe, in the simulations, that the spread of calcium is contained around its entry points, due to the limited diffusion of calcium before extrusion. Our algorithm makes use of this local property to discriminate synaptic activity from other calcium events. Indeed, we observed in simulations typical patterns of synaptic activity (Fig. 5-4B), with a conical shape. These diffusion patterns start at synapses, and propagate to neighboring locations. The typical diffusion length of those patterns, $l_{diff} = 10 \mu m$ was estimated from the simulations. After applying a denoising algorithm to the data (Fig. 5-4A), we observed these typical patterns in the experimental data as well (Fig. 5-4B). We also observed in simulations
‘black regions’, far from synapses (Fig. 5-4B). These black regions are also somewhat observed in experimental data (Fig. 5-4A-B). They are a result of the limited intracellular diffusion of calcium ions. The diffusion of calcium ions coupled to the sensor is negligible since jGCaMP7s is anchored to the plasma membrane. Using these observations, we developed an algorithm to detect synaptic activity in jGCaMP7s-Ca dynamics. The algorithm is based on identifying local spatial maxima of the rising slopes of jGCaMP7s-Ca along the dendritic arbor. Indeed, in our simulations, we observed that the closer a location was to a synapse, the higher the rising slope of the jGCaMP7s-Ca dynamics (Fig. 5-4C). This result is robust, and was observed for different synapses across multiple locations. Hence, to detect synaptic activity in the experimental data, we built an algorithm detecting local maxima in space of the rising slopes (Fig. 5-4D, see Method). Note that in Fig. 5-4D, the local maxima needs to be considered along the arbor topology, and not linearly. In the experimental protocol, four visual stimuli each composed of a 50ms OFF stimulus were applied. To test the robustness of our algorithm, and to investigate the variations of synaptic activity across different individual presentations of the visual stimulus, we applied our algorithm after each stimulus, and compared the localization of synapses (Fig. 5-4D). We display the result of our algorithm for two stimuli, and observe that some synaptic sites are active in both stimuli, and others are active only for a single stimulus (Fig. 5-4D-E).
The rising slopes of stimulus-evoked calcium transients indicate the location of active synapses in simulation and experimental data. (A) Left: structure of a dendritic arbor generated from *in vivo* imaging of a jGCaMP7s-expressing tectal neuron. Right: visual-evoked Ca responses of this neuron, with the position of recorded activity indicated by the colors on the y-axis corresponding to color labels on the neuronal structure. Stimulus times are indicated by the colored arrows on top of the field plot. (B) Left: experimental recordings in the branch indicated with a black arrow in the tree structure in panel A processed through a Bessel filter, demonstrating a typical conical shape of visual-evoked discrete dendritic responses, consistent with simulations of synapses (Right). (C) Rising slopes of the simulated jGCaMP7s-Ca concentration, plotted according to the distance from the closest synapses labeled with magenta stars. (D) Plot of the rising slopes of Ca transients demonstrating how our algorithm identifies local maxima in space across a dendrite in the filtered and normalized fluorescence traces, for two visual stimuli (red and blue arrows in
panel A). Dots represent the slopes at each point at t=60ms following visual stimuli, solid lines represent a fit smoothed in space (see Methods). The positions along the tree of the neuron in A are represented by the color bar in the x-axis. Stars indicate synapses detected by the algorithm.

E: Dendritic arbor as in A, with stars at synaptic locations detected by our algorithm, for the two visual stimuli in D (red and blue, colors correspond).

**Identification of active synapses within the dendritic arbor:** To validate the algorithm presented above (Fig. 5-4), we ran simulations of the full dendritic arbor, with synapses at locations determined by the algorithm. Note that only a part of the dendritic arbor was imaged (Fig. 5-4A). For the rest of the tree with no simulations, we maintained our previous synaptic distribution and organization. As calcium dynamics at synapses is a local behavior, the position of synapses in the rest of the arbor does not influence the dynamics at recorded positions. We then compare the rising slopes between experimental data and simulations (Fig. 5-5A), and observe a very good agreement between them. We emphasize here that we normalized fluorescence data to homogenize the noise before the first stimulus (Method), and this normalization *a priori* is sufficient to generate a good correspondence between the fluorescence amplitude and jGCaMP7s-Ca simulated dynamics (Fig. 5-5). We observe that the decay slope in experiments is well replicated by our model in a subset of the nodes (Fig. 5-5B-C, green and red nodes). We also observe regions with an additional increase in fluorescence-based calcium signal (blue, orange and purple nodes), that we hypothesized may be driven by local calcium-evoked release from intracellular calcium sources within the dendrite.

5.6 **Discussion**
Technological advancements in imaging platforms now allow for recording neurons with unprecedented spatial and temporal resolution (Kazemipour et al., 2019; Sakaki et al., 2020). When combined with advances in calcium sensors (Dana et al., 2019), it has become possible to sample across the complete dendritic arbor and soma of a neuron at rates enabling the detection of fluorescence signals driven by a wide range of calcium sources including synaptic potentials, back-propagating action potentials, voltage-gated calcium channels and endoplasmic reticulum-mediated calcium release. These new datasets raise the challenge of developing methods to segregate these sources for independent analyses. Here, we have created a model to predict synaptic activity and their locations on dendritic arbors from these complex datasets.

An accurate model for calcium, jGCaMP7s and jGCaMP7s-Ca dynamics: We generated a model for voltage propagation and ionic electrodiffusion in the dendritic arbor at the microscale level. This model is based on the Nernst-Planck equation for ionic electrodiffusion, ensuring the precision necessary to reproduce microscale level dynamics. We created a decoupled version of our model, allowing fast simulation in detailed dendritic arbor geometries achieved from in vivo morphometric imaging experiments. To derive the decoupled version, we show that the longitudinal ionic flux can be well approximated by a resistive current ($j = \sigma E$). Our study also emphasizes the role of electrodiffusion in calcium propagation, versus simple diffusion. Hence, in Fig. 5-2D, we observe that the major part of the calcium flux is the electric flux $f_{Ca}^{elec}$ that is also faster than the diffusive flux $f_{Ca}^{diff}$.
Figure 5-5

Comparison between experimental and simulated calcium traces, with synaptic activity determined using our detection algorithm. (A) Comparison between the filtered fluorescence data (dashed lines, normalized) and simulations of jGCaMP7s-Ca dynamics (solid lines) at several locations in the tree. The gray bar represents the time at which the algorithm compares the rising slopes. (B) Positions on the dendritic arbor of the nodes represented in A and C (colors correspond). (C) Comparison between the raw fluorescence data (points), the filtered
data (dashed lines) and the simulated jGCaMP7s concentration (solid lines) in 5 of the 6 nodes presented in A (colors correspond).

Stimulus-evoked jGCaMP7s-Ca fluorescence rising slopes are a marker for synaptic activity: Here, we propose an algorithm that uses the rising slope of stimulus-evoked calcium fluorescence recordings as a marker for synaptic activity. We observe from our simulations that the rising slope of the stimulus-driven jGCaMP7s-Ca influx is strongly dependent on the distance from active synaptic sites, and we employ this measure for determining the locations of active synapses in real datasets that have a high temporal sampling rate (Fig. 5-4). In our jGCaMP7s-Ca experimental recordings we were able to identify predicted active synapse locations by identifying spatial maxima of rising slopes along the dendritic arbor, and the activity at these sites was consistent with recorded retino-tectal synaptic activity (Demas et al., 2012; Engert et al., 2002; Honda et al., 2011). We observe a strong agreement between our model’s predictions of jGCaMP7s-Ca dynamics and fluorescence-based experimental measurements (Fig. 5-5). Indeed, a peak following the stimulus is observed in both the fluorescence traces and the jGCaMP7s-Ca concentration dynamics, and the rising slope and peak amplitude correspond in most nodes (Fig. 5-5A and C).

The differences in the rising slopes of stimulus-induced calcium signals (Fig. 5-5) is due to the different dynamics of the various calcium sources. In our simulations, NMDA receptor channels were added to model calcium entry at synapses, as well as voltage-gated calcium channels (VGCC), to represent calcium entry consequent to a sufficiently high membrane depolarization. The simulation yields stereotypical conical patterns of spatially and temporarily isolated calcium
signals (Fig. 5-4B), characteristic of synaptic activity. This shape is due to a competition between the diffusion of calcium ions entering at a synapse, and free calcium removal from the cytoplasm, through binding on various buffers and extrusion through pumps. This spatial discrimination of the calcium signal is also due to our fluorescent jGCaMP7s sensor being membrane-bound and exhibiting minimal diffusion. Finally, these patterns are also easily observable due to the higher calcium current amplitude at active synapses via NMDA receptors, compared to regions with no active synapses, where the main calcium current is due to VGCCs in our simulations. In the experimental results, despite the inherent noise and additional calcium sources, we observe a similar pattern of stimulus-evoked calcium influx that we interpret as markers of synaptic activity. To detect these conical shapes, our algorithm searches for local maxima in space of the rising slopes. At other locations in the dendritic arbor we also observe calcium patterns that do not appear in simulations (Fig. 5-5, blue, orange and purple nodes). We hypothesize that the observed prolonged stimulus-evoked calcium transients may result from local calcium-evoked release of calcium from intracellular stores that is not currently modeled (Segal & Korkotian, 2014).

Utility of detecting synaptic activity from fluorescence Ca-sensor recordings: Our algorithm detects and localizes synaptic activity from fluorescence recordings, without the need for synaptic markers such as fluorescently-tagged synaptic proteins or tagged intrabodies that target these proteins (Gross et al., 2013). There is substantial utility for a model that is capable of identifying active synapses from fluorescence-based calcium data, since expression of synaptic markers or immunostaining to localize synapses is often not feasible for in vivo experiments. One challenge arises from the limited number of detection channels typically available in most multi-photon imaging systems. As a result, only a small number of different fluorescent markers can be imaged.
concurrently, such as a calcium sensor reporting activity in one channel and a neuronal morphology structural marker in the second channel. Furthermore, even in experiments pairing a synaptic marker with an activity marker, our algorithm would prove useful for discriminating synaptic responses from signals arising other sources.

**Limitations:** One limitation of our model for discriminating synaptic activity from fluorescence-based calcium signals is the requirement that the datasets have a high temporal resolution, since synaptic activity is discriminated based on the faster rise of the slope compared to other calcium sources. We estimate that to be able to accurately identify synaptic activity from jGCaMP7s fluorescence, recordings need to be performed at a rate of approximately 50Hz. Another limitation is the lack of exact experimental measurements for certain biological variables that by their nature are extremely difficult to quantify. For example, it would be extremely challenging to obtain the exact concentration of jGCaMPs7s expressed in neurons *in vivo*, despite the availability of previous research providing estimations of plasmid expression based on the promoter (Dou et al., 2021) due to likely variable amounts of plasmid delivered to each neuron. Consequently, we performed simulations for a range of different concentrations of jGCaMP7s, which indicate that the results were not significantly impacted.

**Future development of the algorithm:** The algorithm presented here is a first attempt to localize synaptic activity from fluorescence data in the full dendritic arbor of a neuron recorded in the intact and awake developing vertebrate brain. The resultant model successfully localizes suspected synaptic activity in an *in vivo* dataset, however there are several potential improvements that can be made. Firstly, while the signal given by our dendritic recordings is spatio-temporal, we expect
some correlation of the noise at a spatial level as well as at the time level, however our denoising algorithm is currently only temporal. To address this we plan to add the spatial component using the model for electrodiffusion. Secondly, we intend to expand the model to be able to discriminate a wider variety of calcium signals in a neuron. In addition to stimulus driven synaptic calcium, neurons in a stimulus-response circuit also have calcium transients from other internal and external sources that have been demonstrated to be biologically relevant. For example, endoplasmic reticulum-based calcium transients are believed to potentially play a role in modulating synaptic plasticity (Segal & Korkotian, 2014), as are back-propagating action potentials (Waters et al., 2005).

**Application of the algorithm to analysis of synaptic inputs:** This algorithm allows for the identification of synaptic inputs in neurons based on evoked calcium. A natural next step is to adapt it to identify synaptic and extrasynaptic inputs based on neurotransmitter input. This is a new possibility in the field due to the development of increasingly sophisticated fluorescence-based neurotransmitter sensors. In particular, the third generation of iGluSnFR sensors has recently been designed specifically to allow for the temporal discrimination of synaptic glutamate from extrasynaptic glutamate (Aggarwal et al., 2022).

**Building an integrated input-output neural model:** Subsequent to the adaptation of the model for the analysis and discrimination of synaptic glutamate, we intend to apply the model to the analysis of experiments in which both a glutamate sensor and a calcium sensor are simultaneously expressed in a single neuron *in vivo*. Thus, we can build a comprehensive model of both
neurotransmitter input and the resultant calcium output and investigate input-output relationships at synapses.

5.7 Conclusion

In this paper, we present a model and algorithm designed to detect possible synaptic activity in in vivo fluorescence-based calcium recordings. Our model is based on the Nernst-Planck system of equations for electrodiffusion, coupled to a capacitive equation representing voltage dynamics at the membrane. We then decoupled our model to allow for fast simulations at the scale of morphometric experiments. Using a dendritic arbor structure derived from experimental data, we simulated realistic dynamics of calcium while it is bound to the jGCaMP7s sensor. Using these simulation results, we identified a typical conical shape of calcium diffusion following its entry at a synaptic site. This conical shaped calcium transient was present in experimental calcium imaging datasets, enabling it to be used as a marker for synaptic activity. We then built an algorithm to automatically detect this marker. Re-running our decoupled model with the synaptic sites detected by our algorithm, we observed a very good agreement between our numerical simulations and the experimental dataset. We also observed calcium transient patterns not yet identified by our algorithm potentially due to variable number and frequency of AP input at individual synapses, or local release of calcium from intracellular stores, which we intend to adapt the model to be able to discriminate between their sources. Our model and algorithm are tools capable of identifying synaptic activity across a dendritic arbor in in vivo fluorescence-based calcium recordings, at the microscale level.
Table 5-1 Simulation parameters for the channels dynamics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_Na$</td>
<td>conductance of $Na^+$-current</td>
<td>120 mS cm$^{-2}$</td>
<td>(Hodgkin and Huxley, 1952)</td>
</tr>
<tr>
<td>$g_{Na,con}$</td>
<td>conductance of $Na^+$-current</td>
<td>$3 \times 120$ mS cm$^{-2}$</td>
<td>(Hodgkin and Huxley, 1952; Bono and Clopath, 2017)</td>
</tr>
<tr>
<td>$E_{Na}$</td>
<td>equilibrium potential of $Na^+$-current</td>
<td>115 mV</td>
<td>(Hodgkin and Huxley, 1952)</td>
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<tr>
<td>$g_K$</td>
<td>conductance of $K^+$-current</td>
<td>36 mS.cm$^{-2}$</td>
<td>(Hodgkin and Huxley, 1952)</td>
</tr>
<tr>
<td>$g_{K,con}$</td>
<td>conductance of $K^+$-current</td>
<td>$3 \times 36$ mS cm$^{-2}$</td>
<td>(Hodgkin and Huxley, 1952; Bono and Clopath, 2017)</td>
</tr>
<tr>
<td>$E_K$</td>
<td>equilibrium potential of $K^+$-current</td>
<td>$-12$ mV</td>
<td>(Hodgkin and Huxley, 1952)</td>
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<tr>
<td>$g_L$</td>
<td>conductance of leak current</td>
<td>0.3 mS.cm$^{-2}$</td>
<td>(Hodgkin and Huxley, 1952)</td>
</tr>
<tr>
<td>$g_{L,con}$</td>
<td>conductance of leak current</td>
<td>$3 \times 0.3$ mS.cm$^{-2}$</td>
<td>(Hodgkin and Huxley, 1952; Bono and Clopath, 2017)</td>
</tr>
<tr>
<td>$E_L$</td>
<td>equilibrium potential of leak current</td>
<td>10.6 mV</td>
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<tr>
<td>$g_{Ca}$</td>
<td>conductance of $Ca^{2+}$-current</td>
<td>14.5 mS.cm$^{-2}$</td>
<td>(Guerrier and Holcman, 2017)</td>
</tr>
<tr>
<td>$E_{Ca}$</td>
<td>equilibrium potential of $Ca^{2+}$-current</td>
<td>115 mV</td>
<td>(Guerrier and Holcman, 2017)</td>
</tr>
<tr>
<td>$\tau_m$</td>
<td>parameter for $Na^+$-current</td>
<td>10 ms</td>
<td>(Hodgkin and Huxley, 1952)</td>
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<tr>
<td>$\theta_m$</td>
<td>parameter for $Na^+$-current</td>
<td>25 mV</td>
<td>(Hodgkin and Huxley, 1952)</td>
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<tr>
<td>$\eta_m$</td>
<td>parameter for $Na^+$-current</td>
<td>4</td>
<td>(Hodgkin and Huxley, 1952)</td>
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<tr>
<td>$\sigma_m$</td>
<td>parameter for $Na^+$-current</td>
<td>18</td>
<td>(Hodgkin and Huxley, 1952)</td>
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<tr>
<td>$\tau_a$</td>
<td>parameter for $K^+$-current</td>
<td>10 ms</td>
<td>(Hodgkin and Huxley, 1952)</td>
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<tr>
<td>$\theta_a$</td>
<td>parameter for $K^+$-current</td>
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<td>$\tau_p$</td>
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<td>1.3 ms</td>
<td>(Guerrier and Holcman, 2017)</td>
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<td>$\theta_p$</td>
<td>parameter for $Ca^{2+}$-current</td>
<td>102 mV</td>
<td>(Guerrier and Holcman, 2017)</td>
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<tr>
<td>$\tau_l$</td>
<td>parameter for $Ca^{2+}$-current</td>
<td>10 ms</td>
<td>(Guerrier and Holcman, 2017)</td>
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<td>$\theta_l$</td>
<td>parameter for $Ca^{2+}$-current</td>
<td>24 mV</td>
<td>(Guerrier and Holcman, 2017)</td>
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<td>$g_N$</td>
<td>NMDA receptor conductance</td>
<td>0.15 mS.cm$^{-2}$</td>
<td>(Koch, 1999)</td>
</tr>
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<td>$E_N$</td>
<td>NMDA receptor equilibrium potential</td>
<td>75 mV</td>
<td>(Koch, 1999)</td>
</tr>
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<td>$\tau_{N.1}$</td>
<td>NMDA receptor time constant</td>
<td>11.5 ms</td>
<td>(Koch, 1999)</td>
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<td>$\tau_{N.2}$</td>
<td>NMDA receptor time constant</td>
<td>0.67 ms</td>
<td>(Koch, 1999)</td>
</tr>
<tr>
<td>$[Mg^{2+}]$</td>
<td>Magnesium block</td>
<td>2 mM</td>
<td>(Koch, 1999)</td>
</tr>
</tbody>
</table>
Chapter 6: Conclusion

6.1 Summary of Aims

My overarching goal during my doctoral work was to understand how individual neurons in an intact developing model animal compute information and to understand how environmental inputs modify this process. To investigate this, I had several objectives: To design, test and validate the tools that would enable fluorescently labeling a single neuron in an awake, in vivo brain and to be able to record the entirety of activity across the complete structure of a single neuron. Secondly, I wanted to apply these tools to investigate how stimuli shape plasticity patterns in both structure and function (and the connection between both) of the complete dendritic arbor of a neuron. Lastly, I wanted to develop a model utilizing these unique datasets that would enable improved analysis of fluorescence-based calcium activity and the localization of synaptic inputs in these neurons.

6.2 Strengths, limitations and future directions

This research project has yielded several novel advances. In contrast to other research in the field where the analysis of the structural and functional plasticity of neurons is generally tied to either in vitro sampling (H. Lee et al., 2016) or limited section of the dendritic arbor in in vivo models (El-Boustani et al., 2018; Kerlin et al., 2019), during my work, I developed the tools to enable sampling fluorescence across large regions of dendritic arbor, up to and including the whole neuron. Furthermore, in contrast with these previous studies, instead of utilizing mature
neuron in an adult animal, where the stimulus-response circuits are already established and stable, I used a developing model where there is a great deal of plasticity. Together, this enabled me to investigate how a neuron becomes tuned based on its inputs and becomes wired into a circuit using an incredibly rich dataset.

I was able to perform experiments utilizing planar scans where calcium fluorescence from whole regions of dendritic arbor totaling in the hundreds of microns could be recorded at over a hundred Hz, allowing for developing a mathematical model that depended on data for validation with both a high sampling rate and a high spatial specificity to be able to distinguish calcium transients across regions several microns apart. I was also able to perform experiments where, with caveats, I could sample across the complete dendritic arbor and this allowed me be able to look at not just changes in structural and functional plasticity at a local level, but across the complete neuron and thus elucidate global patterns of plasticity that occur when neurons becomes wired into a circuit. Significant limitations were tied to the technical challenges of the tools and the imaging platform. Segmented scanning enabled me to sample the complete neural structure at a given time point, at a rate sufficient to record small, isolated calcium transients that we believed to be synaptic at filopodial tips, however several compromises were made to achieve this. Importantly, due to segmented scanning functioning by sampling each third of the neuron independently and in series, this means that for the majority of the neural arbor it was in many cases, not sampled simultaneously with either the soma or the axon of the neuron. This means that it was impossible to directly evaluate individual input and output relationships: I could compare on average the strength of dendritic input and action potential output at a particular timepoint, but I could not directly see what dendritic inputs led to a particular output. This
meant that I was unable to ask questions about what number and patterns of synaptic inputs led to either an action potential output or a failure.

The inherent trait of RAMP microscopy that enables it fast sampling rate, that it is sampling individual points is also a significant limitation. This causes experimental constraints in two ways. The first constraint is the time required to execute the scans. The initial drawing of the entire structure of the neuron after taking the first volume stack is an entirely manual process, where I had to draw every aspect of the neural structure to then be skeletonized into up thousands of points (Fig. 4-19). As this initial drawing step took 45 minutes, after it was complete a second stack would be immediately taken and for the next 10 minutes I would update the arbor structure with any new growth. At each 30 min epoch, I would take another image stack and between 10 minutes updating scan and 10 minutes sampling that is 20 minutes between taking the last stack and the final scan being completed. Given these developing neurons are highly plastic and a key set of experiments I performed on them involved a plasticity inducing visual stimulus, spaced training, there are potentially changes in dendritic arbor structure occurring in that 20 minute period between when the image stack is taken and the last functional scan is completed that would be missed. The second limitation is that since only the points in the volume stack that are sampled are the pre-plotted points that contain the neuron, even minor drift occurring during the scan can result in some or all of the pre-drawn scanning points drifting off the neuron. I ameliorated this in several ways: first by utilizing paralyzed *Xenopus* tadpoles where motion artifacts from respiration are far less than in mammal models, secondly by scanning an 11 pixel line at each scan point and then averaging the data across that line, allowing for a small amount of drift to be compensated for (Fig. 3-3). After the experiment was completed, I was able to validate the quality of the scans by looking at the kymograph of
different points during scanning to see if they were drifting off. However, despite employing these methods, drift occurring during experiments proved to be a continuing challenge with whole experiments failing because too much drift occurred during a scan at a particular epoch to be compensated for and thus rendering gathering a full experimental dataset an arduous process.

One way to both reduce the time between z-stacks, minimizing missing rapid arbor changes while also reducing the impact of drift would be to cut down on the time necessary to update the drawing of the neuron. The most efficient way to do this would be to automate drawing of the neuron. We have an new morphometrics software suite, DYNAMO that is validated on *Xenopus* tectal neurons (Coleman et al., n.d.), so applying it to eliminate the manual drawing currently necessary for RAMP microscopy would dramatically reduce the time spent updating the drawing and thus the time between drawing updates. An added benefit of this is that automated drawing would drastically reduce the 45-minute initial drawing time (Fig. 4-19), which would allow for more scanning epochs to be added to the experiment, since a significant limitation on the maximum length of the experiment is the amount of time the tadpole remains paralyzed and immobile. Through utilizing automated drawing software, like DYNAMO it would be possible to significantly reduce the biggest limitations of RAMP microscopy.

Despite the limitations of RAMP microscopy, I was able to use calcium datasets derived from it to inform and validate a mathematic model of voltage propagation and electrodiffusion through a dendritic arbor that can be applied to localize synaptic inputs. However, technological improvements have the potential to significantly improve both the utility of this model and the model itself. A major limitation of this model is that its ability to localize synaptic inputs from jGCaMP7s fluorescence is limited to datasets where the sampling rate is at least 50 Hz. This means that while planar scans on the RAMP microscope meet this temporal sampling
requirement, whole neuron and segmented scanning do not, limiting the practical utility of the model with this platform to experiments where only a single plane of the neuron is sampled. However, recently, there has been an in vivo fast imaging platform, called Scanned Line Angular Projection (SLAP) created that could be applied to record whole neuron volumes in Xenopus at much faster rates, on the order of over 100Hz (Kazemipour et al., 2019). Furthermore, new fluorescence activity sensors have been developed to utilize these new fast-sampling capabilities, importantly a new generation of iGluSnFR (Aggarwal et al., 2022). Whereas previous generations of iGluSnFR were synaptically excluded and thus made it challenging to distinguish synaptic from extrasynaptically released glutamate, this new series of glutamate sensors is not synaptically excluded and its kinetics are designed to leverage the fast sampling rate of SLAP microscopy to enable distinguishing synaptic from extrasynaptic glutamate. By designing an experiment combining the expression of this new iGluSnFR with jGCaMP7s in Xenopus tectal neurons, it would be possible to generate a dataset that would be ideal for building out the model further, providing ground truth validation of synaptic calcium transients through co-localized synaptic glutamate signals and allowing for the creation of an integrated input-output neural model.

This research project has yielded developments in tools and technologies that I have been able to utilize to further our understanding of how a neuron acts as a unit of computation and how it shifts how it encodes and processes information in response to a change in its inputs. However, these are still not solved questions, and there are still gaps in our understanding of neural encoding, processing and plasticity. Emergent technologies are opening new avenues to investigate these questions and further our understanding of this fundamental aspect of neuroscience at the root of learning, memory and cognition.
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