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# Chapter 1: General Introduction

## A brief history of glial research

The early neuroanatomist Rudolf Virchow was searching for connective tissue in the vertebrate brain when he observed glia in histological sections and postulated that these cells provide static structural support to nervous tissue (Virchow 1856). Believing that the cells he saw “glued” the brain together, Virchow termed them “neuroglia”, and the name stuck. More functions for glia were soon postulated by other neuroanatomists: Camillo Golgi observed that glial cells often contact vasculature, while neurons do not, which led him to suggest that glia convey circulating metabolites to hungry neurons; Santiago Ramón y Cajal suggested that glia may electrically insulate neurons; Wilhelm His suggested that radial glial fibers guide the migration of nascent neurons; and Ernesto Lugaro proposed that glia regulate the synaptic space (reviewed in Somjen 1988).

Subsequent research confirmed and refined these pathologists’ educated guesses; we now know that during nervous system development, glia produce many neurons and enable proper localization of those neurons (**CITE**); additionally, glia myelinate axons and enable ordered synapse formation (**CITE**). After nervous system development reaches steady-state, glia provide metabolic support for neurons and facilitate neuronal function, e.g. by scavenging neurotransmitters around synapses and maintaining ionic homeostasis of the extracellular space (**CITE**). Additionally, there is mounting evidence that glial cells directly excite, inhibit, or alter neuronal activity on a timescale relevant for sensory processing and behavior. (**CITE**). And we can expect to find even more complexity in glial function as we develop new methods for observing the nervous system.

This introductory chapter contains an overview of glial function in the vertebrate nervous system. In the first section I advance a general theory of glial function, or what we should expect of glia given what we know of neurons. Subsequent sections will enumerate different forms and functions of glial cells, with the goal of illustrating both common and variable glial traits. Along the way I will be hindered by some unfortunate conceptualization about cell function and some unfortunate terminology, starting with the term “glial cell”: Some use the term “glial cell” to denote any non-neuronal cell in the nervous system. This definition is convenient for a scientist whose sole interest is neurons. Unfortunately, this definition lumps together cells with important functional differences. Specifically, microglia are termed glia (because they reside in the central nervous system and are not neurons) but these cells are macrophages of mesodermal origin that invade the nervous system early in development (Alliot et al. 1999), while all other glial cells are derived from the same neuroectodermal lineage and are thus truly endemic to the nervous system (Cameron and Rakic 1991). Unsurprisingly, microglia have very distinctive morphology, behavior, and function when compared to the neuroectodermal glia. Accordingly, subsequent use of the term “glia” will denote non-neuronal neuroectodermal cells (also known as “macroglia” or “neuroglia”).

## A theory of glia

To the typical systems neuroscientist, i.e. someone concerned with how networks of neurons create and transform signals, it is not at first obvious why glial cells would be of interest, or, to put it differently, why neurons can’t do everything the brain needs. But how do neurons work? A single spiking neuron propagates signals by leveraging an electrochemical gradient across its membrane; signals between neurons are typically transmitted by release and capture of neurotransmitters in the extracellular space. Neither action potential generation nor synaptic release are sustainable long-term without dynamical processes that maintain homeostasis. But, despite their utter reliance on the state of the extracellular space for signaling, neurons cannot effectively regulate this domain without sacrificing their signaling ability.

Accordingly, I suggest that any complex nervous system composed of spiking neurons needs regulation of the extracellular space to obtain optimal signal processing by neurons, and that glia provide this regulation in vertebrates. I argue for this claim via the following thought experiment: consider a hypothetical “nervous system” of two neurons in close apposition inside a compartment. if the first neuron fires a barrage of spikes, the electrochemical state of the extracellular space will be altered (e.g., the extracellular potassium concentration increases, extracellular calcium decreases, etc.), which in turn affects the excitability of the second neuron, and thus its ability to engage in signal processing. By sharing the extracellular space, the two neurons cannot signal independently, and this problem becomes more severe if more neurons were added to the compartment. But if the neurons are isolated from each other by a non-spiking cell that can sense and regulate the extracellular space, i.e. a paradigmatic glial cell, then the second neuron is isolated from the externalities of the first neuron’s spiking, and the two neurons can engage in signal processing independently and efficiently, albeit at the expense of a more complicated nervous system (two cell types instead of one). The lesson from this thought experiment applies at synapses: the independence of nearby synapses is compromised if neurotransmitter leaks from one synapse to another. This problem is solved if synapses are surrounded by glial processes that scavenge neurotransmitter.

The paradigmatic glia circumscribed in the above thought experiments could be characterized as “supporting” neurons; this is how glia are typically summarized in the broader neuroscience literature, and neuroscience is a decidedly neuron-centric field. But does electrochemically isolating a neuron from its neighbors really “support” that specific neuron? Does facilitating synaptic transmission “support“ a neuron? Maybe, but It seems more apt to say that glial regulation of extracellular space chiefly supports *neuronal signaling*, or nervous system function as a whole, and thus the interaction between glia and neurons is cooperative, toward a common goal. I believe this is more than a semantic quibble – glia have been observed directly modulating neuronal activity, either by altering the extracellular space to push neuronal dynamics into a different state, or by releasing a ligand for neuron-bound receptors (Morquette et al. 2015; Ma et al. 2016a). These results are difficult to understand if we believe that glia exist to support neurons. But if we believe that glia support *nervous system function*, then dynamically altering neuronal activity is entirely consistent with that mandate, and we should expect to find many more instances of glial dynamics shaping neuronal signaling.

## Classes of glial cells

In the following sections I will enumerate different types of glial cells and their functions. There are many morphologically distinct subtypes of glia in adult animals. As a general rule, specialized neuronal circuits feature correspondingly specialized glial cells: the cerebellum contains Bergmann glia; the retina contains Muller glia; the mammalian cortex contains astrocytes, among others. Before I describe these more specialized glial cells, I will introduce radial glia, which have a valid claim to the title of “ur-glia” as they are the most common glial cells across taxa, the first glial cells to appear in vertebrate development, and their morphological and functional properties nearly span the space of traits in which more specialized glia reside.

### Radial glia

#### A note on terminology

As I noted earlier, some of the standard terminology for glial cells is unfortunate. The term “radial glia” is a chief example, as “radial glia” can be used as a *name*, denoting a specific class of cells, or as a *description*, denoting the class of glial cells with radial morphology. Both uses of the term can denote the same cells, but there are glial cells with radial morphology that are not referred to as radial glia. My usage of the term will follow this unfortunate convention.

#### Morphologies of radial glia: radial fibers

Radial glia are the first glial cells in the nervous system, and their morphology reflects this. Radial glial somata reside at the apical (ventricular) surface, and these cells send processes to the basal (pial) surface of the developing neuroepithelium, where they form a lamellar process called an endfoot that defines the basal extent of the neuroepithelium (Figure 1A). Radial glia are proliferative, giving rise to many neurons and glia during development of the nervous system (Malatesta et al. 2000; Johnson et al. 2016). Neurons born from radial glia migrate from the ventricular zone by climbing radial glial fibers toward to the pial surface; at the appropriate distance from the ventricular zone, these neurons detach from glial fibers and integrate into neural circuits (Figure 1B, Rakic 1972). Calcium imaging studies have revealed that neuronal migration in mouse embryos is modulated by extensive Ca2+-mediated signaling within and between radial glia (Weissman et al. 2004; Rash et al. 2016), which indicates that the role of radial glia in neuronal migration goes beyond providing static scaffolding for migrating neurons. Radial glial fibers are a hallmark of the developing mammalian nervous system, but in many non-mammalian taxa such as fish (**cite**), birds (**cite**), and reptiles (**cite**) neurogenic potential persists into adulthood, and these animals also retain radial glia into adulthood. by the presence of radial glial fibers, suggesting that neurogeneration requires, or is at least greatly facilitated, by the presence of radial glia.

#### Morphologies of radial glia: ramifications

In addition to their long fibers that guide neuronal migration, radial glia can form ramifying processes that intermingle with neuronal processes (Figure 1C). These glial processes extensively fill the space around neuronal processes and thus give radial glia access to synapses and the broader extracellular space. Because this morphological trait is highly conserved across different animal species and across subtypes of glia within species, I will devote the next section to a discourse on why I believe ramifying glia (also known as astroglia) are essential to the nervous system.

### Muller glia

In the retina, specialized glia with radial morphology called Müller glia, ramify around neuronal processes in the inner and outer plexiform layers.

### Bergmann glia

In the molecular layer of the cerebellum, Bergmann glia ramify around purkinje cell arbors, surrounding purkinje-parallel fiber synapses.

### Astrocytes

In many brain areas in mammals, radial glia lose their apical-basal polarity during brain maturation. These cells become astrocytes, star-shaped cells that ramify heavily in neuropil. Astrocytes can form endfeet on blood vessels, which gives them an opportunity to bridge the peri-neuronal extracellular space and the vascular system. Astrocytes are primarily studied in adult mice

**Oligodendrocytes**

In many species, a subset of axons are wrapped by glial processes that facilitate rapid conduction of action potentials.

## The category “Astroglia”

### Astroglial marker genes

A cell’s specific functions can be illuminated by the genes it expresses. Radial glia express a set of glial-specific genes (i.e. genes that distinguish them from neurons or neuroepithelial cells), which encode the following proteins:

The intermediate filaments glial fibrillary acid protein (GFAP) and vimentin, which are believed to give glia increased resistance to mechanical stress (Eng et al. 1971; Dahl et al. 1981).

The glutamate-aspartate transporter (GLAST), a high affinity sodium-dependent glutamate transporter (Storck et al. 1992; Malatesta et al. 2000). This protein allows glia to rapidly clear of perisynaptic extracellular glutamate, which facilitates glutamatergic synaptic transmission and prevents neuronal death from glutamate excitotoxicity.

The enzyme glutamine synthetase (GS), which performs ATP-dependent conversion of glutamate to glutamine (Akimoto et al. 1993). Radial glia release glutamine into the extracellular space for neurons to take up and convert to glutamate.

The calcium-binding protein S100B, which can

Brain lipid binding protein (BLBP)

Gap junction protein connexin 43

The water-permeable channel aquaporin 4 (AQP4)

Inward-rectifying potassium channel Kir 4.1

### Observing astroglial dynamics with calcium imaging

Early studies in cultured astrocytes indicated that, like many cells, glia respond to extracellular stimuli by transient increases in intracellular calcium concentration (**CITE**).

Unlike neurons, astroglia do not use fast sodium spikes for signalling. Thus, temporal dynamics of glial activity are impractical or impossible to study with the electrophysiological tools used for monitoring neurons. Instead of firing sodium spikes, astroglia respond to chemical signals via second messenger pathways, most notably transient increases in intracellular Ca2+ concentration. By introducing a calcium sensitive dye into astroglia, or by expressing genetically encoded calcium indicators like GCaMP (Nakai et al. 2001) in astroglia (), changes in intracellular Ca2+ concentration become observable as changes in fluorescence which can be detected with a suitable imaging system.

[Summary of results from calcium imaging of astroglia]

Astroglial calcium activity has been most extensively studied in the cortex of adult mice, where the predominant astroglial cell is the astrocyte. Based on their spatial and temporal properties, sensory-evoked calcium events in these cells can be classified into two categories: fast, local elevations in [Ca2+] that occur independently in micron-scale “microdomains” throughout the fine processes of single cell, and slow, cell-wide elevations in [Ca2+] that often involve the somatic cytosol(cite).

Are astroglia-neuron interactions like those described by Ma. et al the exception or the rule?

From flies and mammals we have evidence that astroglia interact with neurons on a timescale relevant for sensory processing and behavior.

## Zebrafish as model organism for neuroscience

Over the last 30 years, larval zebrafish (*danio rerio*) have emerged as a compelling model for neuroscience research. Use of zebrafish in neuroscience builds on extensive use of zebrafish as a model organism for vertebrate development, and parallels the rapid acceleration of molecular and microscopy tools. The small size and optical accessibility of the larval zebrafish brain makes it an attractive platform for asking open-ended questions about the fish nervous system.

#### Behavioral repertoire of larval zebrafish

Larval zebrafish exhibit a range of locomotor patterns (Budick and O’Malley 2000)

#### Evoking zebrafish behavior using virtual reality

## Light sheet microscopy

Raster-scanning fluorescence microscopy techniques, like confocal or two-photon microscopy, are too slow (frame rate < 30 Hz) to meet our volumetric sampling rate requirement. A coincidence of technologies was necessary to achieve our goal of observing calcium activity in astroglia distributed across the volume of the fish brain (300 x 200 x 800 um) at a sustained rate above 2 Hz. First

An alternative technique is to induce fluorescence at the entire focal plane of the detection objective using a low numerical aperture sheet of excitation light and imaging this excited plane onto a camera. [put a figure depicting this geometry]. This technique, termed light sheet microscopy, enables acquiring an image of a single plane at a rate limited by the acquisition speed of the camera, which for modern cameras can exceed 100 frames per second.

#### Analyzing light sheet microscopy data

Light sheet microscopy datasets are large -- our microscope can generate data at a rate of approximately 800 megabytes per second for many minutes. For calcium imaging, all datasets must undergo the following operations:

artifact removal (per image)

sample motion estimation (per image)

sample motion correction (per image)

fluorescence baseline normalization (per pixel)

The units of the raw camera images are proportional to time-binned photons, but for quantitative calcium imaging we need data in biologically meaningful units, e.g. units of calcium concentration. With a calcium sensor like GCaMP6f it’s impossible to accurately measure the true calcium concentration of a cell; however, we can define a fluorescence value as “baseline” and measure changes in the cell’s fluorescence normalized to that baseline. Thus, the raw fluorescence (in units proportional to binned photons) is transformed via the following equation:

(f - f0) / f0

Ideally this baseline value corresponds to the fluorescence observed when the cell is inactive (supposing we can set criteria for “inactive”). From imaging alone we cannot know when a cell is inactive, so we have to make an estimate. With a perfect, noise-free measurement of a cells’ calcium-modulated fluorescence over time, the best estimate of the fluorescence baseline would be the minimum value of that measurement, and if the imaging conditions are perfect we can be sure that this minimum value is greater than 0. But real data are acquired imperfectly. Sensors have noise, the signal-to-noise ratio of fluorescence can be low, and the total amount of functioning fluorophores decreases over time due to photobleaching. These nuisances can be addressed together by estimating a time-varying baseline via applying a temporal smoothing filter to the data.

The choice of filter is non-trivial. Simple linear filters, e.g. a moving average, will effectively mitigate Poisson noise and have well-defined effects on the power spectrum of a signal but their linearity is a limitation: linear filters mix baseline fluorescence with supra-baseline fluorescence, leading to an inflated estimate of the baseline. Thus, we use a nonlinear smoothing filter that is robust to outliers: a moving percentile filter. For some vector v with elements *vt*, the percentile filter returns the *k*th percentile of the values *v[t - w / 2, t + w / 2].* The percentile *k* is usually set to ~20 to ensure that long periods of cell activity do not bias the baseline estimate; the window length *w* is chosen to be the shortest duration in which a cell can be expected to reach an inactive state *k* percent of the time, which in practice is on order of 5 minutes or so.

Computing a percentile filter with a useful window size for baseline estimation can be quite slow. This becomes acute when one needs to estimate the baseline for ~50 million timeseries (the number of pixels containing signals from the fish brain in a typical experiment). Leveraging the fact that the baseline changes slowly, I wrote a baseline estimation function that allows downsampling of a fluorescence signal before applying the percentile filter. This last step was the key to scaling df/f calculation to large datasets

[Figure demonstrating the practice of baseline estimation on fake data]

Analyzing these data in a timely manner (ideally, before the next series of experiments) requires extensive computing and software infrastructure.

There are several bottlenecks that arise when for processing data at this scale. The first is the amount of random-access memory (RAM) needed: As a rule of thumb when manipulating numerical data, for every *X* GB of data to be processed in RAM, one wants *2X* GB of memory for storing intermediate values. Thus, to comfortably process a 500 GB dataset in memory requires 1 TB of memory, which is outside the limit of any consumer-grade desktop workstation. Even if we had such a workstation, as soon as our dataset grew in size we would encounter the same problems all over again. Instead of trying to do all our computation on a single workstation, a more scalable strategy is to distribute the computation across multiple computers -- a single computer with 1 TB of RAM is prohibitively expensive, but a cluster of 10 computers each with 100 GB of RAM is much more attainable.

When datasets exceed hundreds of gigabytes in total size, computation time

[scalable distributed computation using dask]

[image registration]

[]

# Chapter 2: Volumetric imaging of zebrafish astroglia indicates their functional similarity to mammalian astrocytes

## Abstract

Extensive research in mice suggests that mammalian astroglia, including astrocytes, Muller glia and Bergmann glia, are critical for proper assembly and function of neural circuits throughout the brain. These cells respond to variations in local neuronal activity with transient increases in intracellular calcium, but a full picture of astroglial responses to neuronal activity in nervous system function is only starting to emerge. We report astroglial structures in the larval zebrafish brain that appear morphologically and functionally similar to mammalian astrocytes. These findings suggest that glia-neuron interactions are widespread throughout vertebrates, and establish the larval zebrafish as a model for studying this fundamental aspect of nervous system function.

## Introduction

In the past two decades, larval zebrafish have emerged as a bountiful model for basic neuroscience research, owing largely to the optical accessibility of the larval fish nervous system and the availability of powerful genetic tools for creating transgenic animals. Neuroscience research in larval zebrafish has thus far centered around the role of neurons in processing sensory stimuli and driving behavior; at the same time, essentially no effort has been devoted towards investigating the contribution of glial cells to brain function in larval zebrafish. By contrast, astroglia in mice (astrocytes, Muller glia, Bergmann glia) have been studied extensively, with a particular emphasis on using calcium imaging to observe how these cells dynamically interact with neurons and neuronal circuits. The conspicuous absence of research on zebrafish astroglial dynamics seemed to us a missed opportunity which we could immediately address using our existing tools for volumetric imaging.

In the interest of establishing larval zebrafish as a model system for studying glia-neuron dynamics, we created transgenic larval zebrafish expressing calcium indicators in astroglial cells and used these animals to characterize the basic anatomical, functional, and genetic properties of zebrafish astroglia.

In this chapter, we begin by describing the morphological structure of zebrafish astroglia across the brain. We show that the morphology of larval zebrafish astroglia is consistent with reports of astroglial structure from adult zebrafish, and that larval zebrafish astroglia ramify extensive in neuropil regions. Using volumetric fluorescence imaging, we then found that zebrafish astroglia engage in complex calcium signaling events on multiple spatiotemporal scales. In order to understand the molecular basis of the observed calcium dynamics and morphological structure we obtained RNA sequencing data from zebrafish astroglia. Taken together, the results here indicate that zebrafish astroglia can be considered homologous in many ways to mammalian astrocytes. Note: nomenclature for subtypes of glial cells is at times inconsistent, which affords us some flexibility. Typically, non-myelinating glial cells in zebrafish are labelled “radial glia”, because the gross morphology of these cells is radial. However, we will show that these cells form ramifying processes in neuropil regions, much like mammalian astrocytes, and thus we refer to these cells as astroglia, although they could be equivalently called radial glia.

## Results

### Anatomical structure of zebrafish astroglia

We began by characterizing the structure and distribution of astroglia in transgenic larval zebrafish using fluorescence microscopy. We expressed a variety of fluorescent reporters under the promoter for glial fibrillary acid protein (*gfap*), an intermediate filament with expression restricted to astroglia (Bernardos and Raymond 2006a). Fish used for these experiments were within the range of ages typically used for behavioral experiments i.e., 5 – 9 days past fertilization (dpf). Based on prior reports of astroglial structures in adult zebrafish (Than-Trong and Bally-Cuif 2015) we anticipated that larval astroglia would exhibit a radial morphology, with cell bodies abutting ventricular (apical) surfaces and sending long fibers projecting to pial surfaces, terminating in endfeet. As expected, we consistently observed this motif throughout the hindbrain and midbrain of the larval zebrafish (**fig 1: example cell**). In the spinal cord, astroglial cell bodies are distributed dorso-ventrally along the ventricle (**fig reference**) and project to the nearest basal surface (**fig reference**) but in more rostral segments of the hindbrain, astroglial cell bodies occupy increasingly dorsal ventricular territory and these cells project more ventrally **(fig reference)** – in the extreme case, astroglia with somata abutting the ventral aspect of the upper rhombic lip project nearly parallel with the dorsal-ventral axis (**fig reference**). [why does would the projection angle change? Growth of tissue in the basal plate (neuropil)]. We observed putative astroglial cells throughout the spinal cord, hindbrain, midbrain and forebrain (fig reference);

Crucially, we observed that astroglia ramify extensively in neuropil regions (**fig 1: example cell; fig 2: brain-wide distribution**), which suggests that these cells can sense and respond to neuronal circuit activity.

### Anomalous cells labelled by the *gfap* promoter

In transgenic fish with both cytosolic and nuclear-localized fluorophores under the *gfap* promotor, we observed anomalous dimly labeled cells that we deemed more likely to be nascent neurons rather than astroglia based on the following properties: these anomalous cells do not exhibit expected astroglial morphology or soma localization; these anomalous cells are found in brain areas known to be densely packed with neurons; and these anomalous cells are counter-labelled by the neuronal label *elavl3* **(fig: 2-color cytosolic zoom in)***.* Taken together, this evidencesuggests that many of these anomalous cells are likely neurons, which is not surprising given that radial glia divide asymmetrically to produce neurons (Malatesta et al. 2000; Johnson et al. 2016).

What can explain labelled neurons in a gfap transgenic fish? One possibility is that the neuronal daughter cells inherit fluorescent proteins expressed by their glial progenitors, and these proteins persist until they are degraded. Data on the decay half-life for the fluorophores we used (GCaMP6f, tdTomato) are not available, but the decay half-life of green fluorescent protein (GFP) is reported to be approximately 26 hours in mammalian cells (Corish and Tyler-Smith 1999). If we assume the fluorophores used in the present study are approximately as stable as GFP, then we should expect some fluorophore inheritance to occur, given that larval zebrafish are undergoing active neurogenesis.

Another possible explanation for labelled neurons in the gfap transgenic fish is that the *gfap* promoter may not be perfectly specific to glial cells. This possibility contains a range of sub-possibilities: in a mild case, nascent neurons may express decaying levels of gfap; in an extreme case, there may be mature neurons that express gfap. We cannot distinguish between either of these possibilities using the data presented here.

The gfap promotor may also drive variable expression of fluorophores across subpopulations of astroglia. For example, our gfap transgenic fish have relatively sparse labelling in the optic tectum, while a transgenic fish expressing markers using the her4.1 promoter have much denser labelling of the optic tectum (Image). Researchers studying mouse astrocytes found that the gfap promoter only labels a subpopulation of astrocytes in the mouse brain (**Citation**), and have since largely abandoned use of the gfap promotor in favor of the alcohol dehydrogenase promotor aldh1 (**Citation**). Given these caveats, it is important to bear in mind that a) we cannot be certain that all cells labelled by the gfap promotor are glia, and b) we cannot be certain that all glia are labelled by the gfap promotor.

Nonetheless, our gfap-transgenic animals form a sufficient basis for us to begin studying the calcium dynamics of astroglia in larval zebrafish.

### Multiscale calcium dynamics observed in larval zebrafish astroglia

Astroglial calcium activity spans multiple spatial scales, from compartmentalized opening of few transmembrane calcium channels to ensemble calcium flux in large populations of cells **(Citation)**. Comprehensively observing astroglial calcium activity requires imaging populations of astroglia in their entirety, from end-foot to cell body, over hundreds to thousands of cells. To this end we used light sheet microscopy, which allows rapid, long-term volumetric imaging in transparent samples at resolution sufficient to capture sub-cellular events (Keller et al. 2008; Vladimirov et al. 2014). Using this technique, we observed calcium activity in astroglia across a range of spatial and temporal scales, which is broadly consistent with what other researchers have observed in mammalian astroglia. For the purposes of this chapter, I will decompose these scales into three classes of calcium signaling events: single-cell events, circuit-level events, and brain-wide events; we observe many instances of these classes of events in larval zebrafish astroglia.

#### Single-cell events

From calcium imaging studies of mammalian astrocytes, we know that calcium flux within a single astrocyte can be quite complex.

#### Circuit-level events

and temporal scales, which is broadly consistent with what other researchers have observed in mammalian astroglia. For the purposes of this chapter, I will decompose these scales into three classes of calcium signaling events: subcellular or single-cell events, circuit-level events, and brain-wide events.

#### Brain-wide events

We consistently observed large ensembles of astroglia engaged in spatially propagating waves of calcium activity. In mice, similar phenomena, termed “calcium waves”, been reported in Müller glia (**CITE**), Radial glia (**CITE**), Bergmann glia (Cite), and astrocytes (CITE).

[Simultaneous dual-population imaging]

[History of glia responding to neuromodulators]

[Bergles pape], [nedergaard papes], [ma freeman pape]

## Discussion

## Materials and methods

[optogenetics of glia]

[Why spatially targeted perturbation]

With our microscopy methods we can continuously observe a large number cells in the nervous system, but observation alone is rarely sufficient to resolve the causal relationships in a complex system. Targeted perturbations are usually necessary to convincingly demonstrate that a causal link exists in a complex biological system.

# Chapter 3: A noradrenergic error signal acts through astroglia to suppress futile behavior

## Abstract

When goal directed behavior repeatedly fails, it can be advantageous to reflect on these failures and try a different strategy. We recapitulated this phenomenon in a virtual reality paradigm for larval zebrafish. In this paradigm, fish received a constant drive to swim yet received no visual feedback from their actions. Experience of motor futility, i.e., repeated actions with no sensory feedback, caused fish to alternate between active and passive behavioral states, characterized by vigorous efforts and quiescence, respectively. In order to understand how the nervous system generates this response to behavioral futility, we used brain-wide functional imaging to record calcium signals from the whole nervous system --neurons and astroglia-- in animals as they alternated between active and passive states. We identified a population of astroglia in the hindbrain that appear to drive the transition from active to passive behavioral states; we subsequently identified hindbrain noradrenergic neurons that provide excitatory input to these astroglia, and GABAergic hindbrain neurons that receive excitatory input from the astroglia. We compose these observations in the following model: noradrenergic neurons respond to motor actions that fail to elicit visual feedback; this error signal is temporally integrated by astroglia, which, through some unknown mechanism, excite GABAergic neurons that suppress motor output. In this way, the fish can infer that its recent actions are futile and adapt its behavioral strategy.

## Introduction

Goal-directed behaviors, especially movements, are often continuously adjusted by feedback (CITE WOLPERT). If an agent observes that an action fails to match the target of that action, the agent can use this error signal to update subsequent actions. But what should an agent do if its goal-directed actions continuously fail? This question naturally arises in the context of locomotor behavior: inability to move is a life-threatening risk for most motile animals, and an animal’s ability to move is largely determined by the animal’s environment, over which the animal has limited or no control. Terrestrial animals can be stuck in mud or water; aquatic animals can be washed onto land; many predatory strategies hinge on restricting a prey animal’s locomotion so that prey can be summarily ingested.

These are real challenges for almost any motile animal, so we expected that we could use the larval zebrafish as a model organism for understanding how animals respond when their actions are futile, and how the nervous system coordinates this response. Like many fish, larval zebrafish swim forward in response to forward optic flow. This behavior, called the optomotor response (OMR), helps fish stabilize their position in space by cancelling out externally-generated displacement with internally-generated locomotion (Rock and Smith 1986). The OMR is sufficiently robust that larval zebrafish will perform this behavior in a simple virtual reality (VR) paradigm wherein a fish is paralyzed and its motor commands are inferred by electrophysiological recording of motor neurons in the tail musculature (Ahrens et al. 2012a). A visual stimulus, e.g. forward drifting gratings, is shown to the fish, which evokes an attempt to swim; the corresponding swim signal is used to translate the visual stimulus as if the fish had moved.

This technique enables two experimental opportunities: first, virtual reality experiments allow very tight control over what the animal sees, and how its actions affect what it sees; second, virtual reality behavioral paradigms are compatible with mechanistic physiology experiments, e.g. imaging the nervous system at cellular resolution.

VR paradigms for studying sensorimotor processing in larval zebrafish have a parameter called the *gain* of the virtual reality, which determines how much the visual stimulus moves as a function of the vigor of the animal’s motor output. In order to observe how fish respond when their actions are futile, we designed a very simple VR paradigm where the fish sees constant forward gratings (to engage the OMR) but the gain of the VR is 0, i.e. the visual stimulus is in “open-loop”. We observed that fish in open-loop alternated between two behavioral states: an “active” state, characterized by frequent, vigorous attempts to swim, and a “passive state, where the fish made no attempts to swim.

We next wanted to know how this behavior is generated by the nervous system. Our intuition in this area was guided by the fact that, at a coarse level, the fish adopting a passive state resembled the canonical response of rodents observed in a class of assays that evoke behavioral passivity by inexorable, aversive stimuli, such as the forced swim test (Porsolt et al. 1979) and the tail suspension test (Steru et al. 1985). The behavioral responses of rodents in these assays are sensitive to drugs that alter the function of neuromodulators, in particular norepinephrine (NE), so we became interested in whether the noradrenergic system and its targets could be involved in the behavioral bi-stability we observed in larval zebrafish in open-loop. In mammals, NE potently excites astrocytes (Bekar et al. 2008; Paukert et al. 2014b); astrocytes have also been implicated in state-dependent modulation of neuronal circuits (Wang et al. 2012; Poskanzer and Yuste 2016). Accordingly, we decided to consider astroglia as well as neurons in our search for mediators of the behavioral state switch. Like mammalian astrocytes, zebrafish astroglia engage in extensive calcium signaling, thus, calcium imaging (e.g., via genetically-encoded calcium indicators) seemed a promising means of observing astroglia activity. Light sheet microscopy works well for imaging calcium activity in astroglia or neurons (or both at once, using spectrally separated calcium indicators), and we used this technique to record neuronal and glial calcium activity across the brain in larval zebrafish as they alternated between active and passive behavioral states.

Our first finding from these imaging experiments was a population of astroglia projecting to the lateral medulla oblongata (L-MO) with calcium activity that peaked when the animal transitioned from active to passive states. We subsequently performed a range of bidirectional perturbation experiments which strongly suggested that calcium signals in these L-MO-projecting astroglia are necessary and sufficient for the animal to transition active to passive behavioral states. Our neuronal imaging data suggested that a population of hindbrain noradrenergic neurons may excite the L-MO-projecting astroglia, and subsequent perturbation experiments confirmed this. Refined imaging experiments suggested that these noradrenergic neurons encode a sensorimotor error signal, i.e. the mismatch between motor output and expected visual stimulus. When we combined optogenetic stimulation of astroglia with calcium imaging of neurons to find cells “downstream” of the L-MO astroglia, we identified a population of GABAergic neurons that we believe are sufficient for suppressing motor activity. Taken together, we propose that a circuit with neurons and glia detects behavioral futility and drives the transition from active to passive behavioral states in open-loop conditions.

## Results

#### Futile fictive fish behavior in open-loop virtual reality

To study how fish respond when their actions are futile, we modified the VR behavioral paradigm previously used for studying short-term motor learning (Portugues and Engert 2011; Ahrens et al. 2012a). Fish are shown a forward-drifting grating, which evokes the OMR (Orger et al. 2000). In closed-loop mode, fish have partial control over the visual environment insofar as their actions generate visual feedback, while in open-loop mode the animals have no control and thus their actions are futile (Fig 3.1A). The difference between closed-loop and open-loop VR can be parametrized by the gain of the VR, which is a number in arbitrary units that indicates the scaling between the animal’s fictive actions and the visual feedback those actions generate; when gain is 0, the VR is open-loop. In closed-loop, fish exhibit regular motor output, with a bout rate of~1 Hz, and with stable power per swim bout, as evinced by a sample trace from an example fish (Fig 3.1B). This fictive locomotion is consistent with behavior observed in free-swimming fish performing the OMR (Budick and O’Malley 2000; Severi et al. 2014; Dunn et al. 2016). In open-loop, however, the vigor of the swim bouts increased, leading to an extended epoch of high-vigor swim bouts, which we call an “active” state. After some time, the fish abruptly stopped swimming for an extended epoch, which we termed a “passive” state (Figure 3.1B). Fish alternated between active and passive states for the duration of open-loop (Fig 3.1C, Fig 3.1D). Fish recovered a normal swim pattern if closed-loop was restored (Fig 3.1C), which indicates that the alternation between active and passive states observed during open-loop was a direct and reversible consequence of the loss of visual feedback.

Our belief that the active and passive behavioral states constituted two distinct modes of behavior was bolstered by our observation that in some fish the distribution of inter-swim intervals (ISI) in open-loop was bimodal, with a narrow peak around 1 seconds, corresponding to the short interval between swims in active states; and a wide peak above 5 seconds, corresponding to the long gap between swims that defined the passive state (Fig 3.2A, Fig 3.2B). With a hand-picked threshold of 5 seconds, we used the ISI to classify an epoch as an active state (an epoch when the ISI is less than 5 seconds) or a passive state (any ISI with length greater than 5 seconds) (Fig 3.2A). Over 74 fish recorded in the open-loop paradigm, the typical active state lasted 34.2 ± 3.4 seconds and the typical passive state lasted 22.8 ± 2.5 seconds (mean ± standard error of the mean) (Fig 3.2C).

We next examined the finer structure of the behavior in the active state leading up to the onset of passivity, hoping to find some changes in motor behavior that could allow us to sharpen our nascent hypotheses about the neural basis of the behavioral state switch. We found that in the last few swim bouts before passivity, fish attempted to swim more vigorously (Fig 3.3A). Additionally, the ISI increased leading up to passivity (Fig 3.3B); fish also attempted to make more turns before become passive (Fig 3.3C). Because we observed heightened motor vigor leading up to passivity and because we assumed that fish would find open-loop aversive, we checked whether the onset of passivity was preceded by struggles. Struggles are characterized by forceful tail undulations which propagate in a caudal-to-rostral direction (reversed relative to forward swimming) and serves to propel the fish backwards (Liao and Fetcho 2008). We did not find evidence that the onset of passivity is typically preceded by struggles (Fig 3.4A-C).

The terms “active state” and “passive state” are unfortunate insofar as they encourage unwarranted assumptions about the global behavioral state of the animal. We assess the activeness and passiveness of an animal by binarizing one kind of behavior (swimming) while our experimentally expedient use of paralysis prevents us from observing other behaviors, such as jaw or eye movement. The most direct approach would be to implement our open-loop assay in a VR paradigm for unparalyzed fish, as other research groups have done for optomotor behavior (Jouary et al. 2016; Orger and Portugues 2016). This would allow us to monitor a much larger set of behaviors with higher resolution. In lieu of a new paradigm, we can attempt imperfect inferences about hidden behaviors via functional imaging. Specifically, we hypothesized that fish in the open-loop paradigm would engage in a higher rate of spontaneous eye movement during passivity. This would be consistent with a theory wherein fish use an epoch of behavioral passivity as an opportunity to make observations of their environment without interference from vigorous actions. We imaged the calcium activity in neurons in the abducens nucleus, a brain region that coordinates movement of the eyes, in a single fish in our open-loop VR. We observed suppressed abducens activity during active states and elevated abducens activity during passive states (Fig 3.5A), which is consistent with the theory advanced above.

Taken together, our analyses of futile fictive fish behavior in the active state show that the transition from active to passive states is anticipated by gradual changes in the motor pattern of the fish – swim vigor and ISI increase, and fish attempt to turn more before becoming passive. [More stuff tbd]

#### Calcium imaging reveals a population of astroglia excited at the onset of behavioral passivity

In order to search for the cellular basis underlying the alternation between active and passive behavioral states, we turned to brain-wide functional imaging light sheet microscopy. This technique allows us to image calcium dynamics in neurons and astroglia across the brain at ~2.5 whole-brain volumes per second while animals perform fictive behavior in a VR (Vladimirov et al. 2014). A 2.5 Hz volumetric sampling rate is slow compared to rapid changes in brain activity, which may occur on the timescale of milliseconds (Budick and OMalley 2000; Faber et al. 2006), but since the alternation between active and passive states occurs on the timescale of tens of seconds, we believed that we could still resolve the underlying changes in brain activity at a low temporal sampling rate. Specifically, we suspected that the transition from active to passive behavioral states was driven in part by some integrative process which occurred on the timescale of seconds, and this phenomenon should be easily detectable at a relatively low temporal sampling rate.

We imaged calcium activity in neurons, as labelled by calcium indicators expressed under the control of the neuronal promoter *elavl3* (Park et al. 2000), and astroglia, as labelled by calcium indicators expressed under the control of the astroglial promoter *gfap* (Bernardos and Raymond 2006b). For experiments in which we wanted to observe astroglial and neuronal activity simultaneously, we created double-transgenic fish which expressed green and red calcium indicators in neurons and astroglia, and we imaged these animals using a commercial dual-color detection assembly (Fig 3.X).

We made our first observations at a relatively low level of detail – we used dual-population imaging to look at the average calcium activity of all astroglia and all neurons as the fish transitioned from active to passive behavioral states. Unsurprisingly, population neuronal activity was highest when the animal was most active, and comparatively low when the animal was in the passive state (Fig 3.X). To our surprise, the trial-averaged population astroglial activity appeared ramp up prior to and peak shortly after the animal entered the passive state (Fig 3.X). This was our first indication that astroglia may play some role in the transition from active to passive behavioral states.

The averaged activity of an entire cell type is a very coarse level of description – it yields a single timeseries from a diverse population of neurons or astroglia. Under the assumption that single neurons and single astroglia are the fundamental elements which generate our imaging data, the ideal level of description for an imaging dataset would be to have one timeseries per cell. But we have neither the the spatial resolution nor the computational method required for generating this level of description from functional imaging of all astroglia and all neurons with a cytosolic calcium indicator. To approach a more refined description of the data, we used a matrix factorization approach to segment the data into spatially contiguous collections of pixels with similar temporal dynamics. By aggregating pixels into “superpixels”, we greatly reduce the size of a dataset while preserving fine-scale temporal dynamics of interest. This strategy is essentially an application of a standard technique for image segmentation, but in the time domain (Shi and Malik 2000; Felzenszwalb and Huttenlocher 2004). We chose the size of our superpixels to approximately match the assumed size of a cell body. After segmentation, we had a collection of cellular-scale superpixels. This is still a very large dataset, but it was now small enough that we could use it as the basis for inferring functional networks, defined as spatially contiguous collections of superpixels with coordinated activity patterns. In the same way that we used matrix factorization to combine pixels into superpixels based on the similarity of their timeseries, we used matrix factorization again to cluster superpixels into putative functional networks based on their timeseries. In order to make conclusions that could be easily compared across a group of fish, we performed the multiscale matrix factorization on multiple fish, and these fish were registered to a common anatomical atlas; we then screened for putative functional networks that were shared across multiple fish, and finally we looked at how these putative functional networks responded when fish transitioned from the active state to the passive state. We found multiple putative functional networks that showed consistent activity patterns near the onset of passivity, including neuronal and astroglial networks with elevated activity in the passive period (Fig 3.X). We devised a response reliability measure to quantify how consistently each network responded to the behavioral state switch. This analysis highlighted an astroglial network localized to a gliapil region in the lateral hindbrain, which we termed the lateral medulla oblongata, or L-MO (Fig 3.X), wherein calcium activity reliably ramped during active states and peaked at the onset of the passive state. For higher spatial resolution, we repeated the reliability analysis on individual superpixels and found consistent results: astroglial superpixels in the L-MO responded most reliably at the onset of passivity.

[Possibly insert some details about the L-MO that one can infer from Z-Brain atlas]. [Say something about the morphology of the astroglia that project to the L-MO]

#### Bidirectional perturbation of L-MO-projecting astroglia suggests a causal role in the onset of behavioral passivity

Our analysis of the brain-wide imaging data led us to suspect that calcium signals in astroglia in the L-MO were playing a causal role in the transition from active to passive behavioral states; specifically, we suspected that elevated calcium signaling in these astroglial cells was somehow causing the fish to stop swimming. The ideal experiments for testing this hypothesis require tools for bidirectionally modulating calcium excitability in only the L-MO-projecting astroglia with high temporal precision; unfortunately, tools satisfying these requirements do not currently exist, so we performed a series of perturbation experiments using a variety suboptimal, albeit extant, tools. We started with a series of perturbations designed to extinguish calcium activity in L-MO astroglia, with the hypothesis that these perturbations would subsequently reduce the probability of fish entering the passive state while in open-loop VR, i.e. that astroglial calcium activity is necessary for the active-passive state transition. We assumed that if we killed the astroglia projecting to the L-MO by ablating their cell bodies, then any intracellular calcium signaling in the L-MO will be extinct. We created transgenic animals with a green fluorophore localized to the nuclei of astroglia (*gfap*:h2b-gcamp) and used an ultrafast pulsed laser to ablate the nuclei of L-MO projecting astroglia (Tsai et al. 2009). We then compared the behavior of these animals in the open-loop VR assay before and after astroglial ablation. After astroglial ablation, the amount of time the fish spent in the passive state was reduced by half relative to before ablation (Fig 3.X). Killing hundreds of cells suddenly (on a cellular timescale) in the central nervous system is likely to have many off-target effects, such as triggering an immune response and possibly damaging untargeted cells. Additionally, astroglia are likely important for maintaining homeostasis of the extracellular space; therefore, abruptly removing astroglia would likely shift or disrupt a wide range of cellular processes. Thus, it is possible that direct side-effects of ablation, or secondary effects due to missing astroglia, could somehow affect neuronal circuits that control motor behavior and thereby generate the post-ablation behavioral phenotype we observed. It is difficult to test this alternative hypothesis directly, so we performed a series of assays designed to measure whether astroglial ablation caused non-specific changes to the fish’s motor behavior. When we measured the behavior of the animals in closed-loop VR after astroglial ablation, we observed normal fictive behavior: after astroglial ablation, fish could still cease swimming when the OMR-inducing stimulus abated, and fish could still adjust their motor vigor to compensate for changes in the gain of the VR (Fig 3.X). We also examined the open-field behavior of unparalyzed fish before and after astroglial ablation, and observed that astroglial ablations had no effect on swim vigor, frequency, or velocity (Fig 3.X). Furthermore, we compared neuronal calcium activity in the L-MO before and after astroglial ablation, to check whether there were changes in neuronal dynamics in the region directly affected by the loss of astroglia, but we found no detectable difference in neuronal activity (Fig 3.X). We also tried a gentler perturbation: in order to specifically block release of calcium from internal stores, we puffed the inositol 1,4,5-trisphosphate receptor (IP3R) blocker Xestospongin C (XeC) on the cell bodies of L-MO-projecting astroglia. Like the ablation of astroglia, blocking IP3R-mediated calcium signaling reduced the amount of time fish spent in the passive state while in open-loop VR (Fig 3.X).

We next performed as series of perturbations in order to test whether elevating calcium activity in L-MO-projecting astroglia was sufficient to induce a passive behavioral phenotype. We first used a chemogenetic approach to tonically elevate astroglial calcium levels in behaving animals. To perform this manipulation, we expressing the rat TRPV1 channel under the *gfap* promotor. Rat TRPV1, but not the endogenous zebrafish TRPV1, becomes highly permeable to calcium when it binds the molecule capsaicin. We used a sparse expression strategy where only a fraction of glial cells expressed the channel because we were concerned that a gfap:TRPV1 transgenic might result in neurons expressing TRPV1, which would add a major confound to any behavioral phenotype observed after TRPV1 activation in these animals. Our sparse expression strategy – injection of embryos with DNA and a transposase -- resulted in TRPV1 expression in a fraction of astroglial cells. Despite the small number of astroglia expressing TRPV1, addition of capsaicin to the fish water triggered elevated calcium activity across the entire population of astroglia in the central nervous system (Figure or movie), suggesting some intercellular communication between astroglia whereby transgenic cells could locally excite non-transgenic cells, e.g. gap junctions (Scemes and Giaume 2006). To test whether TRPV1-mediated elevation of calcium activity could induce behavioral passivity, we measured fictive behavior of sparse gfap:TRPV1 transgenic fish in a closed-loop VR. Specifically, we quantified the fraction of time the fish spent a passive behavioral state. At baseline, i.e. before capsaicin was added to the bath, fish were passive 2.3±1.2% of the time; after capsaicin was added, fish were passive over 60% of the time (Figure 3.X). Neither saline administered to gfap:TRPV1 fish, nor capsaicin administered to wild-type fish, was sufficient to induce a comparable change in behavior.

This result is consistent with our hypothesis that elevated calcium activity in astroglia is sufficient to induce passivity, but the tonic elevation in astroglial calcium induced by capsaicin is phenomenologically distinct from the temporally sparse calcium activity we observed in the open-loop assay, and we ultimately excited astroglia across the central nervous system, rather than just the L-MO-projecting astroglia. Thus, we endeavored to find a way to elevate astroglial calcium with more temporal and spatial precision. Fortunately, light-activated ion channels designed for depolarizing neurons can, if expressed in astroglia, increase astroglial calcium concentration to mimic physiological activation (Figueiredo et al. 2014; Cho et al. 2016). We expressed the short-wavelength channelrhodopsin CoChR (Klapoetke et al. 2014) under the *gfap* promoter, which gave us the ability to excite these cells with blue light. We integrated a digital micromirror device (DMD) into our VR setup, which allowed us to direct excitation light to a region of interest with high spatiotemporal resolution (Zhu et al. 2012b). After using calcium imaging to validate that optical excitation of astroglia evoked physiologically plausible calcium responses, we recorded fictive behavior from gfap:CoChR fish in a closed-loop VR assay and observed the effect of acute photostimulation of astroglial processes in the L-MO region. Optically exciting astroglial processes in the L-MO induced strong, transient reduction in fictive swimming (Fig 3.X), which is consistent with our hypothesis that these cells cause the fish to become passive in open loop. A drawback of using CoChR is that it would also excite any neurons that happen to be labelled in our *gfap* transgenic (Sloan and Barres 2014). We addressed this drawback by repeating these experiments with the optogenetic G-protein-coupled receptor Opto-α1-AR, which, when activated, drives release of calcium from intracellular stores, without directly modulating the membrane potential like a channelrhodopsin (Airan et al. 2009). Stimulating astroglia using Opto-α1-AR expressed under the *gfap* promoter also suppressed swimming in closed-loop VR (Fig 3.X). Taken together, these perturbation experiments provided strong evidence for our hypothesis that astroglial activity directly contributes to the onset of passivity in open-loop VR.

#### Putative GABAergic neurons may receive excitatory input from L-MO astroglia

We found it very unlikely that astroglial activity in the L-MO was sufficient on its own to change the behavioral state of the animals. Rather, we believed that L-MO-projecting astroglia influence behavior by influencing on local neuronal circuits, as has been observed in other model organisms (Ma et al. 2016b). Our brain-wide imaging data revealed that a portion of the L-MO neuropil reliably activated at the onset of passivity (Fig 3.X). By simultaneously imaging calcium activity in neurons while optogenetically exciting astroglia, we found that putative GABAergic neurons in the L-MO showed elevated calcium activity after astroglia were excited (Fig 3.X). Furthermore, optogenetically activating these putative GABAergic neurons suppressed fictive swimming (Fig 3.X), much like the astroglial excitation.

#### L-MO astroglia are excited by norepinephrine

After observed that astroglia are excited during the transition from active to passive behavioral states, we immediately suspected that the astroglia were responding to norephinephrine (NE) released from the noradrenergic system. This suspicion came from two directions: first, studies of astroglia in other animals have found that NE is a potent driver of astroglial calcium responses (Paukert et al. 2014b)

**The noradrenergic system conveys swim failures to radial astrocytes**

Futility-induced passivity results from multiple motor failures, which must be communicated to radial astrocytes by neurons. We hypothesized that the noradrenergic (NE) system might drive radial astrocytic Ca2+ signals based on known relationships of NE to behavior (Bouret and Sara 2004; Aston-Jones and Cohen 2005; Sara 2009; Tervo et al. 2014) and its effect on astrocytes (Bekar et al. 2008; Ding et al. 2013; Paukert et al. 2014a; Ma et al. 2016b; Slezak et al. 2018; Stobart et al. 2018). We found that neurons in the locus coeruleus (LC) and the noradrenergic cluster of the medulla oblongata (NE-MO; Figure 6A, Figure S6A; Farrar et al., 2018; Tay et al., 2011) increased activity before switches to passivity. We imaged Ca2+ in NE neurons and glia simultaneously (in *Tg(th-P2A-Gal4)* (Li et al., 2015)*; Tg(UAS:GCaMP6s); Tg(gfap:jRGECO1b)*). During futility-induced passivity, signals from NE neurons and glia were temporally coupled, with NE cell responses preceding astrocytic Ca2+ (Figure 6B,C; Video S8).

Radial astrocytes express multiple NE receptors (RNA sequencing, Table S3; *in situ* hybridization, Figure S6B), and NE axons project to L-MO (expansion microscopy, Figure S6C; Video S9), showing that glial processes in L-MO could be driven by NE neurons.

Ablating NE-MO greatly reduced time spent in passivity, but no significant reduction resulted from ablation of the LC or dorsal raphe nucleus (DRN) (Figure 6D-F). Thus, the NE-MO, which may be homologous to NE cluster A2 in mammals (Rinaman 2011), is necessary for futility-induced passivity.

To directly test if NE-MO activates radial astrocytes and triggers passivity we optogenetically activated NE-MO while imaging radial astrocytes (Figure 6G; Zhu et al., 2012a) in fish expressing CoChR in NE cells and jRGECO1b in radial astrocytes (*Tg(dbh:KalTA4); Tg(UAS:CoChR-eGFP); Tg(gfap:jRGECO1b)*). This led to Ca2+ responses in glial processes in L-MO and induced passivity for ~10 seconds (Figure 6H,I). In fish with radial astrocytes ablated (as in Figure 4A-C), activation did not lead to passivity (Figure 6I; Figure S6E), showing that passivity resulting from artificial NE-MO activation also requires radial astrocytes.

We identified the noradrenergic receptor that activates radial astrocytes (RNA sequencing data in Table S3). After adding prazosin (α1-adrenergic receptor antagonist, 50 µM) or cyclazosin (α1B receptor antagonist, 10 µM), optogenetic activation of NE-MO no longer caused radial astrocyte activation (Figure 6J-L). Puffing α1 agonist elicited Ca2+ responses and blocking the α2 receptor did not eliminate Ca2+ signals (Figure S6F-G). Thus, L-MO radial astrocytes respond to NE through the α1B adrenergic receptor.

Finally, we tested whether astrocytic Ca2+ responses were driven solely by NE, or by a combination of NE and local circuit activity like mammalian astrocytes in visual cortex (Paukert et al. 2014a). We used swimming, which correlates with L-MO neural activity, as a proxy for local circuit activity (Figure 6M). Activating NE-MO during swimming caused a greater glial response than activating NE-MO without swimming (Figure 6N). Thus, radial astrocytic Ca2+ integrates NE and local circuit activity.

## Discussion

A behavioral strategy for handling these dire encounters would be highly adaptive in the evolutionary sense. Suppose an immobilized animal uses the feedback-stabilized motor control strategy that works well under normal locomotion. While immobilized, all locomotion attempts fail to move the animal and thus they will produce error signals; if the animal responds to these error signals by raising the vigor of its subsequent efforts, then the animal will soon be attempting to move with maximum vigor, yet these vigorous actions will still generate error signals. The feedback-stabilized motor control strategy fails here: it equilibrates with the animal exerting maximum vigor for every action, yet accomplishing nothing, or worse. Generally speaking, vigorous actions are more metabolically expensive, more salient to potential predators, and more likely to interfere with observation of the environment than less vigorous actions; as long as these vigorous actions fail to remobilize the animal, their costs weigh over the nonexistent benefits. These costs can all be minimized by ceasing all efforts as long for as those efforts are futile, i.e. by adopting a passive behavioral state, where the urge to move is suppressed. The animal should only become passive once it can infer that its recent actions have accomplished nothing and thus that the same holds for subsequent actions. But if the animal wants to remobilize, this passive state cannot last forever – the animal should be prepared to act again at the first sign that it has regained mobility, and sometimes the simplest way to test for remobilization is just to attempt to move again. Under these conditions, the animal can be expected to alternate between active behavioral states, which maximize the short-term probability of escaping immobilization, and passive behavioral states, which minimize the long-term probability of metabolic depletion and exposure to predators. In summary, immobilization, due to a predator or environmental conditions, is a life-threatening failure mode for feedback-stabilized motor control. The failure mode can be addressed by adding a higher-order control rule: when repeated movement attempts fail to produce the correct feedback, become passive for some interval of time before attempting to move again.

## Materials and methods

Fictive swimming in virtual reality

Paralyzed larval zebrafish attempt to swim in response to forward optic flow despite their inability to generate motion. When fish are paralyzed with a technique that blocks the neuromuscular junction, e.g. the nicotinic acetylcholine receptor antagonist alpha-bungarotoxin, motor neurons in the tail of the fish will still fire when the fish attempts to swim. The extracellular voltage change associated with motor neuron activity can be recorded with a suction electrode, and this signal can be used to cancel the optic flow

[Strategies for targeted perturbation]

There are several techniques available for spatially patterned light delivery. A simple, direct approach is to use a dual-axis galvanometer to raster-scan a laser beam in the desired pattern. A downside of this approach is the sequential nature of the raster-scanning, which adds a delay between the first and last point scanned; for many applications (including ours), optical stimulation must be synchronous across the stimulation area.

An alternative approach is to use a spatial light modulator (SLM) to generate an arbitrary intensity pattern which can then be optically delivered to the sample. SLMs are pixel arrays that can rapidly modulate either the amplitude or phase of incident light. The fastest, simplest, and cheapest amplitude-varying SLMs are digital micromirror devices (DMDs): arrays of tiny (~10 micron diagonal) mirrors that can rapidly rotate in plane to direct light toward a target (“On”) or toward a beam dump (“Off”).

DMDs have been used extensively for patterned photostimulation (Zhu et al. 2012a). Typically the optical design of such photostimulation systems is optimized for projecting a sharp image. Optically, this entails illuminating the DMD chip with high-NA light (i.e., the widest range of angles which the collection optics can accept). Optimizing lateral resolution comes at the expense of axial resolution—in other words, a sharp projected pattern will have a very short depth of focus. [Figure showing high vs low NA patterned illumination]. For our application (stimulating relatively large volumes of neuronal and glial tissue) we wanted the projected pattern to stay in focus over a long axial range, so we needed to illuminate the DMD chip with low NA light.

There are commercial patterned photostimulation modules [cite polygon and nikon?] but we decided against these solutions for three reasons: first, the price of these systems is prohibitive (in excess of $10,000); second, commercial systems do not meet our optical requirements, and third there is no guarantee that a commercial system will have software that is simple to integrate with our existing behavioral paradigm software.

Software design for photostimulation experiments:

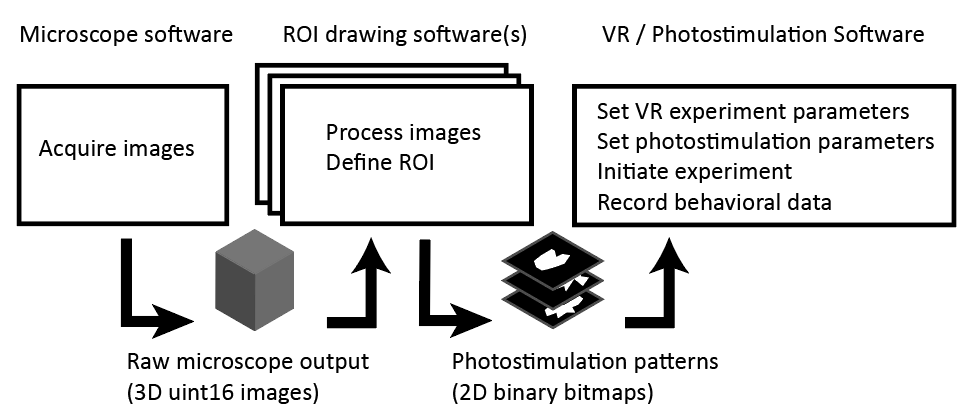
After assembling the optical hardware, I needed to build software that would allow experimenters (starting with me) to effectively use the hardware. At this point I had to make a careful decision about what software infrastructure to build.

I needed software to enable two operations: a) creation of ROI for photostimulation, and b) running the photostimulation experiment (i.e., displaying ROI, activating lasers, etc.). Should these two functions be features of an integrated piece of software, or should I write separate software for ROI creation and experiment execution?

I decided to go for the latter option, chiefly because I anticipated that different experimenters may use wildly different software methods for defining ROI. For some experiments (such as mine) it is sufficient to draw ROI manually, based on the experimenter’s prior knowledge of anatomy. For other experiments, ROI may be determined algorithmically, e.g. as output of analysis of a functional imaging dataset. At the same time, I did not anticipate that different experiments would require drastically varied approaches to executing a photostimulation experiment.

So, I decided that the software for generating ROI would yield bitmap images as output, and the software for running the experiment would take these bitmaps as input. Using bitmap images to represent ROI is convenient because the bitmap format is standard, (i.e. bitmap files are easy to read, write and inspect); additionally, storing ROI as files (as opposed to using a purely in-memory structure) provides self-documentation to an experiment.

I implemented the image preprocessing and ROI drawing pipeline in a Jupyter Notebook (Kluyver et al. 2016) which combines executable Python code, data visualization, and formatted text in a single document. This environment is ideal for experimenters rapidly iterating on an experiment.

[Photostimulation experiment workflow] 

# Chapter 4: A low-cost extensible microscopy platform for volumetric imaging in behaving larval zebrafish

## Abstract

The larval zebrafish retains an optically accessible nervous system over a developmental timeframe, during which the animal develops increasingly complex behaviors (Fero et al. 2011). To leverage both these traits in a single experiment, several groups have designed frameworks for brain-wide imaging of larval zebrafish while the animals are engaged in behavioral tasks (Ahrens et al. 2012, Vladimirov et al. 2014, Panier et al. 2013). Our lab previously published a design for an experimental apparatus combining a light sheet microscope (for rapid volumetric calcium imaging or, more recently, voltage imaging) with an electrophysiology-based closed-loop virtual-reality (VR) behavioral paradigm. Here we report the evolution of our previously published design – a cheaper, simpler, more expandable system for volumetric imaging in behaving larval zebrafish.

## Introduction

Our microscope is built around two hard constraints: The first constraint is that the sample (a zebrafish larva) must be oriented in its natural posture, i.e. with its ventral surface pointing toward earth. Thus, to image the brain, the detection objective must be oriented vertically. The second constraint is that there must be sufficient free space below the sample so that a projector can project a visual stimulus on the bottom of the sample chamber (where the fish can detect it). Since there are no commercial options which satisfy these two constraints, we must design and build our own systems, the most recent of which we present here.

## Results

### Basic capabilities

Our light sheet microscope has two orthogonal excitation arms which each form a light sheet by rapidly scanning a low-NA Gaussian beam. Each excitation arm features a dual-axis galvanometer; one scan axis is used for generating the light sheet, the other axis is used for translating the light sheet up or down to match the focal plane of the detection objective. Volumetric imaging is performed by rapidly translating the detection objective with a piezo flexure positioner while coaxially translating the light sheets. We use two cameras separated by a dichroic beamsplitter for simultaneous detection of spectrally distinct fluorophores, e.g. GCaMP6f and jRGECO1b. The fish is held on an acrylic pedestal in a custom-fabricated water-filled chamber with glass walls. This chamber has a diffusive screen on the bottom for projection of a visual stimulus.

### Ergonomics

In order to accommodate more space for detection optics, we fold the detection path by 90 degrees immediately after the detection objective; thus, all our detection optics can lie on a large, horizontal breadboard. This allows us to comfortably fit two cameras for simultaneous two-color imaging, with ample room remaining for additional optical modules. This horizontal breadboard stands on four optical rails, which also support the two orthogonal excitation arms.

For our VR behavioral paradigm, we attach suction electrodes to the tail of the fish immediately before imaging. This procedure is performed with the aid of a stereomicroscope, and thus the sample chamber must be clear of the excitation and detection optics while the suction electrodes are being attached. Once the suction electrodes are attached, the sample chamber and the electrophysiology equipment (suction electrode, micromanipulator, etc.) must be smoothly translated to the space under the detection objective. To enable this smooth translation, we mounted the fine positioning stages and the sample holder assembly on a manual linear bearing assembly that can be locked in place once the sample is under the detection objective. This manual stage is faster, cheaper, and easier to operate than an electronic alternative.

### Optics

#### Excitation

Like our earlier design, we use two orthogonal light sheets to comprehensively access the larval fish brain. One light sheet propagates along the medial-lateral axis of the fish brain; however, the fish’s opaque eyes block the light sheet from illuminating tissue between the eyes. To illuminate this volume, we use a second light sheet that propagates along the rostro-caudal axis of the fish. In our new design these two excitation arms are assembled in 60mm optical cage, which facilitates alignment of optical parts and introduction of new optical elements into the excitation path.

#### Detection

### Electrophysiology

We replaced the Axon Instruments Axoclamp 200b ($20k) with the Intan RHD2000 evaluation module. The Intan amplifier offers the same performance for our application (recording motor neuron axons from the tail musculature of the fish) with a greatly reduced cost and physical footprint. We designed and fabricated a compact 3D printed housing for the Intan amplifier chip (supplementary materials reference).

## Discussion

Light sheet microscopy (LSM) is not the only method for fast functional imaging in larval zebrafish: raster-scanning two-photon microscopy (TPM) can image at higher resolution and at greater depth in tissue than LSM, but the relatively low efficiency of two-photon excitation limits the data acquisition of TPM rate to a fraction of that attainable with one-photon methods. On the high end in terms of acquisition speed is light field microscopy (LFM), a technique which allows imaging a volume of tissue in a single camera frame. But the speed of LFM comes at a price: lateral and axial resolution are inhomogeneous and low compared to LSM; furthermore, the volume snapshots acquired in LFM must be computationally post-processed before data can be visualized in (*x*,*y*,*z*) spatial coordinates. For our immediate purposes LSM strikes the ideal balance between speed and resolution, but should we need either higher imaging speed or better tissue penetration (or both), then it is straightforward to add either TPM of LFM functionality to the system we describe here.

## Materials and methods

# Chapter 5: Time- and space-efficient baseline calculation for large functional imaging datasets

## Abstract

Constant advances in bioimaging tools enable researchers to image biologically relevant phenomena faster, longer, and at higher resolution. In turn, imaging experiments are producing larger and larger datasets, which pose unique challenges for data analysis due to their scale. These challenges are particularly acute for calcium imaging experiments using light sheet microscopy: these datasets are often so large that necessary image processing must be parallelized using cluster computing. Parallelizing functions on images is straightforward, and many tools exist to enable this. However, analyzing calcium imaging data requires *first* parallelizing functions on images, *then* parallelizing functions on time series, and the required context switch is computationally cumbersome and hard to scale. We present a strategy for dynamically estimating an essential quantity – baseline fluorescence – from calcium imaging time series without the computationally expensive context switch, and we show how this strategy results in faster, scalable, and more flexible processing of large volumetric fluorescence data.

## Introduction

Functional fluorescence imaging via light sheet microscopy is a powerful method for observing dynamics in large populations of excitable cells. In the ideal scenario, an experimenter collecting functional imaging data would have easy access to biologically meaningful results from the data with the minimum effort and latency. However, both the computationally demanding nature of functional imaging datasets and their size pose barriers to rapid analytics.

### Preprocessing functional imaging data

Parsing functional imaging data is intrinsically computationally demanding: for functional imaging data, raw images alone are not highly informative without extensive preprocessing. Intensity values in raw images indicate the quantity of excited fluorophores, but the units of interest for functional imaging are *percent change over baseline fluorescence*, or Δ*f* / *f*0, where *f0*denotes baseline fluorescence and Δ*f* denotes the difference between the raw fluorescence and *f0*. Without normalizing by the fluorescence baseline, inactive cells that express a large quantity of fluorophore appear brighter than dimmer, more dynamic cells. After calculating Δ*f* / *f*0, those silent cells will appear dim while the fluctuations of active cells will be bright **(Fig 5.1C)**. Converting a raw fluorescence image to a Δ*f* / *f*0 image requires estimating the baseline fluorescence *f*0, which is a time-domain statistic estimated by aggregation of potentially hundreds of other images (time points). A further complication is that the baseline fluorescence is often modeled as a time-varying quantity -- *f*0 becomes *f*0(t) -- in order to compensate for slow changes in the fluorophore concentration, e.g. due to photobleaching. Done this way, a single baseline-normalized image can require a large amount of computation, and performing baseline-normalization for an entire dataset requires proportionally more computation. An additional complication is that movement of the sample that occurred during image acquisition will produce strong artifacts after baseline normalization, so it is best to correct for sample motion *before* baseline normalization, e.g. by generating a template image and shifting each image to best match the template. Both estimating and correcting sample motion are computationally intensive. So, thinking concretely about an experimenter who has just collected a functional imaging dataset: before the experimenter can see the data in the units of interest (Δ*f* / *f*0), each image must be first motion-corrected and then normalized by the baseline fluorescence **(Fig 5.1a-c)**. For small datasets, e.g. those that fit in random-access memory (RAM) on a single workstation, these computations may take time but don’t require extensive data engineering; but when datasets become extremely large, these computations can become extremely burdensome to the extent that significant that new computational tools are needed.

Figure 1: Schematized preprocessing for functional imaging data. A) In raw data, silent cells are brighter than dynamic cells, and sample motion along with photobleaching generates spurious dynamic signals from ROIs (colored circles). After motion correction and baseline normalization, dynamic cells are much more prominent than silent cells and spurious dynamic signals from sample motion and photobleaching have been removed.

### The unusually high bandwidth of light sheet microscopy

Light sheet microscopes acquire 4 megapixel images at up to 100 Hz, which corresponds to a peak data rate of ~820 megabytes (MB) per second, per camera. At this rate, the volume of data collected in 20 minutes (typical for a functional imaging experiment) will easily exceed the memory capacity of a high-performance computer workstation. The rate at which light sheet microscopes generate data is more than an order of magnitude greater than that of pre-existing functional imaging modalities. Raster-scanning microscopes with resonant galvanometers acquire 0.26 megapixel images at 20-30Hz, corresponding to a peak data rate of ~15 MB per second, per detector, or 1/50th the peak data rate of a light sheet microscope. Datasets from raster-scanning microscopes can be processed entirely on a single workstation, but the same is not true for light sheet microscopy datasets -- because the data rate of light sheet microscopy is so much higher than for previously established imaging modalities, new tools and techniques are needed for processing the data.

### Computational strategies for processing large imaging datasets

Image processing often involves repeating the same algorithm or computation on different parts of a dataset. When these computations are independent of one another, then they can be processed in parallel, assuming there are sufficient computational resources to do so. Light sheet microscopy datasets present ample opportunity to leverage parallelized computation. Sample motion correction can be independently estimated for each time point, and thus this computation can be parallelized across the time axis of the data. Baseline normalization can be independently performed for each pixel, so this computation can also be parallelized, but across the space axes of the data. So, how should we implement these parallelizable computations? I will first discuss two general approaches to parallel computing, ordered by increasing complexity: first, running preprocessing code on a powerful single workstation; second, running preprocessing code on a compute cluster. I will then schematically outline the existing approach for preprocessing light sheet functional imaging data on a compute cluster, and identify limitations of this approach that will be addressed by the present work.

#### Parallel image processing on a single workstation

The simplest approach is to run preprocessing software on a single workstation with a large number of central processing unit (CPU) cores, each of which supports independent computation; as of this writing, it is possible to purchase workstations with tens or hundreds of independent CPU cores. Besides the simplicity of working on a single computer, a strength of this approach is that analysis software can be written in such a way that all CPU cores can access the same pool of memory.

However, this approach has a fundamental drawback: one cannot scale up the compute capacity of a single workstation. Once a program saturates all CPU cores on workstation, there is no way to dynamically add more capacity. This limitation is particularly unfortunate for functional imaging preprocessing, where the datasets, and thus the concomitant computational load, are constantly growing larger.

#### Parallel image processing on a compute cluster

An established solution to the scaling problems described above is to run programs on a distributed system of many interconnected computers sharing a single file system (a “compute cluster”). This paradigm, termed “distributed computing”, enables dynamically scaling computational resources beyond the limits of a single workstation – on a compute cluster, it’s trivial to harness thousands of CPU cores to execute computations in parallel. But this scalability comes at the expense of a more complex and restrictive programming model. Because the cluster is composed of many separate computers, software designed to work in this context cannot assume that all CPU cores share the same memory space. But for extensively parallelizable problems that are CPU-limited (like most of the preprocessing required for functional imaging datasets) the lack of shared memory is dwarfed by the huge gains in scalability. Parallel programs designed to run on a distributed computing infrastructure can, if designed properly, also run on a single workstation with multiple cores, but the inverse is not necessarily true: a parallel program designed to run on a single workstation (with a shared memory pool) may be very difficult to transfer to a distributed computing environment. Given this asymmetry, and in the interest of generality, subsequent discussion will assume that parallel computations are executed on individual CPU cores in a distributed context, i.e. without the assumption of a shared memory pool. Also in the interest of generality I will hereafter use the term “worker” instead of “CPU core” to denote a unit capable of independent parallel computation. In the following section I will describe a simple workflow for performing vital preprocessing (motion correction and baseline normalization) of functional imaging data in parallel. Without any optimizations, this workflow works for relatively small datasets, but it scales poorly and has some deep inefficiencies, which I will address later in this work.

#### Parallel preprocessing for functional imaging

Functional imaging preprocessing typically begins with operations that are independent for each image, such as applying denoising filters, applying geometrical transformations to images to correct geometrical distortion induced by data acquisition, as well as estimating and or correcting for sample motion. Because these operations are independent for each image, they can be easily parallelized in a scheme where each worker loads an image (or collection of images) from storage and processes them accordingly (**Fig 5.2a**). When the dataset is distributed in this way, the spatial axes of the dataset are local to each worker and the temporal axis is distributed across workers (**Fig 5.2a**). After these various per-image operations, the data must be baseline-normalized. Like motion correction, baseline normalization is also parallelizable computation, but along a different axis of the data: for baseline normalization, the computation is independent for each *time series*, i.e. each the spatial temporal axis of the data needs to be local to each worker, while the spatial axes must be distributed across workers (**Fig 5.2c**). Between distributed processing of images and distributed processing of time series the allocation of data to the workers must drastically change. This procedure, termed “data repartitioning”, requires transfer of data between workers, e.g. by each worker writing and reading data to a shared file system (**Fig 5.2b**). Once data have been repartitioned and the dataset is distributed along the spatial axes, each worker can perform baseline normalization in parallel (**Fig 5.1c**). At this point the baseline-normalized time series can be saved to disk, collected to a single workstation, or re-repartitioned into images for tasks that require parallel processing of baseline-normalized images (e.g., making a maximum z-projection for each time point) (**Fig 5.2d**).

The workflow I have just described has two major problems. First is the data repartitioning step between image processing and time series processing (**Fig 5.2b**). Data repartitioning between workers is slow and brittle – reading and writing many small files to disk is slow, and a single crashed worker during repartitioning can be fatal to the entire process. Additionally, the complexity of data repartitioning grows supra-linearly with data size. Thus, any strategy to avoid data repartitioning is desirable for efficient and scalable data processing of large imaging datasets. Second, because of the time-domain implementation of baseline normalization, getting a single baseline-normalized image at some time index *t* strongly depends on images in a range of time indices above and below *t*. For functional imaging in larval zebrafish, a single baseline-normalized image depends on a temporal window of hundred images. So, in addition to the time required for data repartitioning, there is also a substantial temporal cost due to loading hundreds of images from disk just to yield a single image as output.

In order to mitigate these problems, we report a technique, termed baseline compression (BC), that avoids excessive data repartitioning in distributed functional imaging preprocessing workflows by exploiting the slow temporal structure of the fluorescence baseline. instead of estimating the baseline fluorescence for every time point, we instead estimate the baseline for a sparse, evenly-distributed subset of time points, and then save this down sampled baseline to disk. For subsequent baseline normalization, the baseline for every time point can be estimated independently by interpolating the compressed baseline, e.g. via loading a few images from disk. Thus, via baseline compression, we can avoid the inter-image dependencies and concomitant data repartitioning present in the simple parallel preprocessing workflow presented above. The result is faster, more stable preprocessing of functional imaging data, but this comes at the expense of accuracy.

## Results

Given the limitations inherent to the parallel preprocessing workflow presented earlier, I was motivated to find a way to dynamically calculate baseline normalization without extensive data repartitioning, i.e. a way to perform baseline normalization independently for each image. A conceptually simple solution would be to compute the full baseline once, save the resulting baseline images to disk – i.e., complete the computational graph shown in **Fig 5.2** -- then load the baseline images as needed for subsequent normalization (**Fig 5.3a**). However, in practice this approach would demand extensive data duplication, which may be undesirable – for each image, the baseline of that image would need to be saved to disk, thereby doubling (at a minimum) the storage footprint of the dataset. However, if we could generate a *compressed* version of the fluorescence baseline and save it to disk, we could then use this compressed baseline to generate reconstructed individual baseline images when needed without duplicating the entire dataset. These reconstructed baseline images could then be used for normalizing the corresponding raw fluorescence images. How could we compress the baseline? The fluorescence baseline varies slowly in time, as the chief drivers of baseline fluctuations are photobleaching and changes in cell state, both of which occur at a relatively slow timescale. With this in mind, it should be possible to represent the dynamics of the full baseline by downsampling and interpolating between a relatively small number of time points. Thus, we decided to test the following procedure: first, for each pixel, calculate the baseline fluorescence at a small set of evenly distributed time points, and save these points as well as the subsampled baseline to disk in a file format that allows fast reading from arbitrary array regions (e.g., raw binary or hdf5). Then, to convert a raw image from time index *t* to a baseline-normalized image, we load the baseline images *i* and *i + 1,* where *i* denotes the index of the subsampled time point that precedes time index *t.* To linearly interpolate, a weighted average of the two baseline images is computed based on the relative position of raw image time index *t* in the interval *(i, i + 1)*. The resulting reconstructed baseline image is then used to divisively normalize the raw image.

As a proof of concept, I compared the full fluorescence baseline (estimated using a sliding window percentile filter) with a downsampled baseline (estimated by downsampling the sliding window baseline by a factor of 250; 1 subsampled time point every150 s) for a few sample pixels from a light sheet microscopy dataset wherein the whole brain of a larval zebrafish (expressing the calcium indicator GCaMP6f under the pan-neuronal promotor *elavl3*) was imaged at 1.7 Hz (**Figure 5.3.1**). Single pixels are the smallest and noisiest elements in a light sheet imaging dataset, and any downstream analysis pipeline of light sheet data will involve aggregation of data across many pixels; therefore, any large systematic errors observed at this stage would be a warning sign. Fortunately, this initial diagnostic indicates that the full baseline and the downsampled baselines are qualitatively very similar, without large systematic errors, regardless of whether the pixels collected fluorescence emitted from an active cell or inactive tissue (**Figure 5.3.2**). The full baselines are very slowly varying, likely due to the very low rates of photobleaching in by light sheet microscopy. Because the baselines vary so slowly, a relatively small number of points is required for a veridical reconstruction.

[Insert figure 5.3.2]

Continuing the qualitative evaluation, I next considered every pixel in the ROI that contained the four pixels displayed in Figure **5.3**. It would not be possible to display the full baseline and the downsampled baseline for each pixel for comparison, so instead I performed baseline normalization using the full baseline and the downsampled baseline for every pixel in the ROI and examined a sample frame, the max intensity over time, and the minimum intensity over time. For all these image-domain diagnostics, the baseline-normalized images look qualitatively identical regardless of whether the full or downsampled baseline was used for normalization. This again suggests that the downsampled baseline, once reconstructed, is a workable approximation of the sliding window baseline.

**[Insert 5.3.2]**

Having demonstrated on small datasets that the fluorescence baseline can be compressed through downsampling, the next step was to scale up the procedure to larger datasets and consider the reconstruction error across a large number of pixels. Instead of calculating the full sliding window baseline for each pixel and then downsampling it (as I did in figure **5.3**), I devised a scalable technique for calculating the downsampled baseline directly, which enabled me to generate a host of simple diagnostics for evaluating the downsampled baseline technique on large datasets.

In order to estimate a useful range of downsampling factors for each dataset, I computed the time-average of the entire dataset and estimated the full baseline of that time series, yielding an “average baseline” **(Figure 5.X.X)**. I then downsampled this average baseline with a range of downsampling factors and plotted the normalized mean error between the full baseline and each downsampled baseline **(Figure 5.X.X)**. By examining these plots, one can make a partially informed decision about how much downsampling (and thus how much reconstruction error) is acceptable for the full dataset. For example, from the plot in **5.X.X**, one can see that the error is low and stable for baseline sampling intervals less than ~180 seconds.

Now that I had a heuristic for picking baseline sampling rates, I wanted to know the mean and worst-case reconstruction error for each pixel in a large dataset, as well as spatial maps of these errors, all as a function of the degree of baseline downsampling, and for different types of samples. These are all important statistics for an experimenter or analyst who wants to use the downsampled baseline technique. First, I looked at a single plane from the same volumetric imaging dataset that served as the source for the data in **Figure** **5.3**. For each pixel, and for a range of downsampling factors, I computed the sliding-window baseline and the downsampled baseline and measured two quantities: the time-averaged normalized error between the sliding-window baseline and the downsampled baseline (computed as the time average of the normalized error: ) and the maximum absolute normalized error (computed as the maximum over time of the absolute value of the normalized error).

When the mean normalized errors across all pixels are visualized as a histogram, it becomes apparent that the mean error distribution is centered around 0, and the vast majority of pixels have mean normalized error under 1% for all levels of downsampling tested, and the highest mean normalized error is approximately 2% (**Figure 5.4.1**). The worst-case errors also look acceptable across a wide range of baseline sampling intervals, with a median maximum error consistently around 5% (**Figure 5.4.1**). More frequent sampling of the baseline does seem to strongly contract the long tail of the maximum error distribution, but this is driven by only a very small number (100-200) of pixels. Spatial maps of the mean error (**Figure 5.5.2**) generatedfor **XS** downsampling condition,show that mean error does not form clusters around anatomical structures, which is reassuring, and consistent with the relatively flat fluorescence baselines in the data. The spatial map of the maximum error (**Figure 5.5.3**) does show anatomical clustering – error is relatively high around a few cells in the optic tectum, and in the area postrema. In both of these brain regions reconstruction error is high due to phenomena that defy our model of the slowly varying fluorescence baseline – [describe cell moving into the plane and whatever phenomenon is occurring in the area postrema]. The conclusion of these diagnostics is that, across a wide range of downsampling values, the reconstructed baselines have very low mean and maximum error.

[Glia now]

To show the generality of this approach, I repeated the same diagnostics on data from a different excitable cell type and a different fluorescent reporter – astroglia expressing the calcium indicator jRGECO1a. As we saw for the neurons expressing GCaMP6f, the mean and maximum error rates are quite low across a wide range of sampling intervals (**Figure 5.6**). The maximum error distribution for the astroglial cells has a much shorter tail, meaning that there are no instances where the baseline fluorescence fluctuates dramatically.

To demonstrate the utility of the downsampled baseline technique to data from organisms beyond larval zebrafish, I next repeated the prior diagnostics on data from GCaMP-expressing neurons in the explanted central nervous system of a *drosophila melanogaster* larva. In

## Discussion

I have demonstrated a technique for rapid, scalable baseline normalization for large functional imaging datasets.

[Baseline fluorescence estimation]

[Other elaborations of this technique]

Baseline compression by downsampling and reconstruction by linear interpolation is deliberately simple, but it is likely that other techniques would produce lower reconstruction error. For example, using cubic spline interpolation instead of linear interpolation for reconstruction would ensure that the reconstructed baseline is differentiable and likely reduce reconstruction error, at the expense of greater computational complexity in reconstruction and a larger file size for the compressed baseline. Temporal downsampling as a compression technique is also relatively crude, compared to standard time series compression methods. Higher compression ratios and lower reconstruction errors are likely attainable by using frequency-domain techniques like fourier-domain or wavelet-based compression schemes (**wavelet cite**), although performant random access to individual baseline image would likely require a much more complicated data structure than the one I designed for temporal downsampling compression.

[How people can be expected to use the technique]

Error is inevitable in any approximation. In this particular case, the downsampled baseline technique allows a data analyst to trade off error for speed. Although the error rate attained via the downsampled baseline was quite low across all three datasets, even a 1% error may be too high for some analyses. I do not claim that the lossy compression scheme presented here is a complete substitute for a baseline estimate that uses all available temporal information. What the downsampled baseline technique offers, however, is an accelerated path through the intermediate phase of a research project, between raw data and a final result. The compressed baseline technique enables a researcher to rapidly home in on cells or regions of interest in a dataset, and then potentially study those areas with the increased accuracy of the full baseline, which can be easily calculated for small chunks of data.

[Where this technique fits in the bigger picture]

Functional imaging with a light sheet microscope can comprehensively record activity from large populations of excitable cells over a long period of time. But the promise of this technique can only be realized with new techniques for processing these enormous datasets.

## Materials and Methods

Imaging data of larval zebrafish were collected on a custom-built light sheet microscope (Vladimirov et al. 2014). Single volumetric time points were saved to disk as unstructured binary files, one per time point. Raw data files were each converted to hdf5 format before being transferred to the network file system (NFS) at Janelia Research Campus.

Imaging data of *drosophila melanogaster* larval explant were collected on a separate custom-built light sheet microscope (**CITE**). [explain Keller lab post-processing, figure out what the genotype the fly was] .

Distributed computing was performed using the high-performance compute cluster at Janelia Research Campus. The compute cluster is divided into nodes, each of which has up to 48 CPU cores

[InterpArray data structure]

In the implementation presented here, the downsampled baseline is represented as an array with the same spatial dimensions as the original dataset but with a contracted temporal dimension. Through a function (linear interpolation), we can generate a one-to-one mapping between elements in the raw dataset and elements in the downsampled baseline. In order to reify this abstraction, and to simplify usage of the downsampled baseline technique in analysis code, I designed a data structure that enables an interpolation-augmented representation of a downsampled array.

Reconstructing a baseline image requires linearly interpolation at most two consecutive elements from the downsampled baseline array.

# Chapter 5

## Concluding remarks

### Chapter 2

### Chapter 3

### Chapter 4

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