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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).

[**Known roles for glia**]

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 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 enumerate different forms and functions of glial cells. A note on the meaning of the term “glial cell”: Some use the term “glial cell” to denote any non-neuronal cell in the nervous system. 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”).

## 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.

I will note (here and wherever else it is appropriate) that some of the standard terminology for glial cells is unfortunate. As one example, “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. My usage of the term will follow this inconsistent convention.

### Radial glia

#### Morphologies of radial glia: radial fibers

The earliest glial cells in the vertebrate brain are radial glia, which send processes from the apical (ventricular) surface 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 ne

#### 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.

#### The importance of extracellular space in the nervous system

Why might it be important for glia to have access to synapses and the broader extracellular space? To answer this question, we should consider how 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 (and neither action potential generation nor synaptic release are sustainable long-term without dynamical processes that maintain homeostasis). Despite their utter reliance on the state of the extracellular space for signaling, neurons cannot effectively regulate this domain. 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. My argument for this claim requires 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 would only become 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. The same 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. 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. 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. 2016). If we believe that glia exist to support neurons, then these results are difficult to understand. But if we believe that glia support *nervous system function*, then dynamically altering neuronal activity is entirely consistent with that mandate.

### Muller glia

In the retina, specialized glia with radial morphology called Muller 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 2006). 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)**. Therefore, 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 at resolution sufficient to capture sub-cellular events (CITE). By comparison to other imaging methods, light sheet microscopy is uniquely suitable for imaging calcium dynamics in large populations of cells: raster-scanning methods like confocal and two-photon microscopy are too slow

[spontaneous calcium activity in zebrafish astroglia]

define a filter that picks out single-cell events

frequency of single cells getting activated

spatial distribution of localized calcium events

[sensory- and behavior-evoked calcium activity]

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).

[brain-wide glial calcium events]

spatial source of astroglial calcium waves

frequency of astroglial calcium waves

propagation speed of astroglial calcium waves

behavioral correlates of astroglial calcium waves

[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.

[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) the 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. 2012). 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:

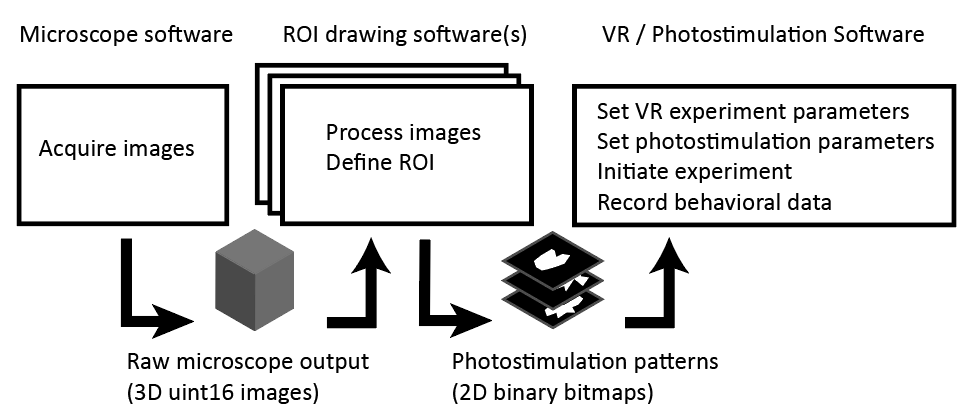
After assembling the optical hardware, I needed to build software that would allow experimenters (starting with me) to effectively use the hardware. At this point I had to make a careful decision about what software infrastructure to build.

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

## Abstract

## Introduction

## Results

## Discussion

## Materials and methods

# 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.

In order to test whether the fluorescence baseline of active neurons could be downsampled with, I first tested it on a few cells from a light sheet imaging dataset.

## Discussion

## 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

# Concluding remarks

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