Overall - Specific Aims

The general goal of the proposal is to generate a realistic multiscale circuit model of the larval zebrafish brain – the Multiscale Virtual Fish (MVF). The model will span spatial ranges from the nanoscale synaptic level, through local microcircuits, all the way to inter-area connectivity. The purpose is to explain and simulate the quantitative and qualitative nature of an animal's behavioral output across multiple spatial and temporal scales. While the work will be focused on zebrafish, our proposal incorporates comparison to other model systems, specifically *Drosophila* and rat. These systems have unique and complementary strengths that will allow us to extend our studies in zebrafish and identify general principles of neural function. To accomplish our goals we will address the following specific aims:

Aim 1: Behavioral analysis, whole-brain imaging and modeling to describe five ethologically conserved behaviors. In pilot studies (our previous U01 project), we successfully integrated quantitative behavioral assays with whole-brain imaging, whole-brain electron microscopy (EM) and theory, to develop a comprehensive framework for the optomotor response (OMR). We propose to extend and expand the OMR project with four additional ethologically relevant behaviors: phototaxis, rheotaxis, escape and hunting. To this end, we will first extract the precise algorithms underlying each behavior and subsequently develop a version of the circuit model to understand its neural implementation. All five of these models will be combined into a unified model containing elements that are shared across - or unique to - the five individual paradigms.

Aim 2: Decision making in the context of conflicting stimuli. Many, if not all, of the five behaviors in Aim 1 will interact in a complex fashion when the stimuli that trigger them individually are presented simultaneously – as is often the case for natural behaviors under real-world conditions. For example, a whole-field leftward moving stimulus may drive the fish to execute a left turn (OMR) while an increase in brightness on the right side may induce a right turn (phototaxis). We will probe several combinations of these interactions at the behavioral level, and measure and analyze underlying neural activity under such conflicting conditions. The ultimate goal is to extend and refine our model of the virtual fish by incorporating experimental findings on multimodal integration and decision making.

Aim 3: Modulation of behaviors by internal states. All individual behaviors, as well as their interactions, depend on internal states, such as hunger, stress or loneliness; which in turn are subject to contextual conditions such as starvation, ambient UV irradiation and social deprivation. These slow, modulatory brain states are linked to neuromodulatory systems such as serotonin, acetylcholine, epinephrine and dopamine, which are already well described in the larval zebrafish. In the third aim, we will characterize such internal brain states, and examine how they influence and modulate the specific behaviors described in Aim 1, as well as the interactions described in Aim 2. An intermediate goal of Aim 3 is to uncover the correlative relationship between internal states (e.g,. such as those that cause hunger or stress) and the activity in various brain areas (e.g., dorsal raphe, hypothalamus). Notably, nutritional deprivation causes modulation of sensorimotor transformations across all species. As such, hunger is an ideal internal state to investigate in the context of efforts to extend our findings to other organisms such as the fruitfly larva and the rat (see Project 4 for details).

Pilot studies and preliminary work towards achieving Aim 1 are already in progress and we expect several, if not all, of the new behavioral assays to be incorporated into the Multiscale Virtual Fish (MVF) within the first five years. Similarly we expect, within that timeframe, to make significant progress towards the goals outlined in Aim 2. To this end, our established experimental infrastructure allows the study of interactions between conflicting trigger stimuli at the behavioral and whole-brain imaging levels, and will further allow us to generate new neural circuit-level insights into sensorimotor processing and decision making. We envision that adding the representation of internal states to the MVF, as outlined in Aim 3, as well as firmly establishing unifying principles across model systems, as proposed in Project 4, will extend the timeframe beyond the first five years.

Understanding the emergent properties of the brain requires a deep understanding of its components, and detailed, biologically accurate brain simulations offer the opportunity to answer fundamental questions about how these properties arise. Here we propose to integrate the current knowledge available in the larval zebrafish, and to complement it by a series of targeted experiments, to give a detailed and holistic description of how complex behaviors arise from the synaptic to the whole brain level in an integrated fashion.

Overall-Research Strategy

The three main aims of the proposal are to develop a comprehensive circuit description that can account for 1) several ethologically conserved behaviors 2) decision making processes in the context of conflicting stimuli and 3) the role of internal states in modulating these processes. We propose to achieve these three main aims by integrating four (sub) projects, each of which is considered instrumental for the successful completion of each aim. Our insights from the larval zebrafish, *Danio rerio* (the fish), will be integrated with two other important model organisms, *Drosophila melanogaster* larvae (the fly) and the rodent, *Rattus rattus* (the rat), to provide a common framework for addressing similar questions across diverse animals.

Finally, our projects are well aligned with six out of the ***seven BRAIN High Priority Areas (BRAIN HPA 1 - 7)*** for the brain initiative (BRAIN 2025, a Scientific Vision, https://www.braininitiative.nih.gov/2025/), as indicated in each project title. HPA 6 - Advancing Human Neuroscience - is the only one not explicitly covered within our projects.

The Projects

1) **Project 1 - Atlas (*HPA 1 - Discovering Diversity and HPA 2 - Maps at Multiple Scales)*:** the generation, curation and publication of an online atlas of the larval zebrafish. This atlas will be based on the existing framework of the Z-Brain [6], and it will include all anatomical regions of the fish brain at three different resolutions: 1) Macro - interactive 3D visualization of all major brain regions and nuclei. 2) Micro - light microscopy data for annotation of cell types and projections, calcium imaging for a quantitative description of functionally defined cell types and regions. 3) Nano - EM microscopy for nanoscale resolution. We believe that such a database, containing a quantitative description of all of these factors, is an essential basis for the generation, verification and refinement of realistic circuit models. (Lead: Lichtman)

2) **Project 2 - Behavior and Imaging (*HPA 3 - the Brain in Action and HPA 4 - Demonstrating Causality)*:** five ethologically important behaviors will be probed in freely swimming and tethered zebrafish larvae to extract the underlying algorithms. This will be combined with functional brain-wide calcium imaging to inform circuit models, as well as with optogenetics and sparse EM reconstruction of physiologically identified circuits to test and validate the models. (Lead: Engert)

3) **Project 3 - Modeling and Theory (*HPA 5 - Identifying fundamental principles)*:** the ultimate goal of the proposal is the generation of a realistic, multiscale circuit model – the Multiscale Virtual Fish (MVF). To facilitate the generation and validation of the circuit models, a software system for integrated simulation and analysis will be developed. It will handle anatomy data as well as functional data and will allow for short model – simulate – inspect cycles in order to rapidly judge the quality of the proposed circuit models. The model will be refined by constant dialogue between modelers and experimentalists. Sparse reconstruction by EM of selected neurons previously identified by calcium imaging, as well as circuit visualization through viral tracing and optogenetics assisted perturbations, allows us to constrain and verify the proposed synaptic connectivity of the model. (Lead: Sompolinsky)

4) **Project 4 - Other Model Systems/General Principles (*HPA 5 - Identifying fundamental principles)*:** To elucidate how brains in general process sensory information and guide behavior, we will integrate our work into two ongoing research projects on *Drosophila* larvae and rats. We will closely align the work on all three model systems and develop common experimental and analytical frameworks to facilitate cross-species comparisons. Specific examples on issues in sensory processing, decision-making, and neuromodulation where interesting parallels already exist across all three species are outlined in the companion document. A central feature of Project 4 is to force researchers studying different animal models to convene regularly and often to compare results and cross-illuminate vexing problems in each system. We believe this trans-species team approach is essential to ferreting out general behavioral principles that ultimately have relevance to human behavior. (Leads: Samuel and Ölveczky)

Combined, the four projects aim to integrate new technological and conceptual approaches produced in HPA 1-5 to discover how dynamic patterns of neural activity are transformed into perception and action which is the essence of overall HPA 7: From BRAIN Initiative to the brain. The projects are roughly defined by the expertise of the five main PIs - and we note that each of the three proposed aims requires the synergistic efforts described in all four projects. All projects are explained in detail in supplemental documents “Project 1 – 4”.

The Core Facilities

In order to carry out these projects, we propose the creation of five core facilities that will provide the necessary resources for the research program.

1. Administrative Core (located at the Center for Brain Science (CBS) in Northwest Labs Building. Lead: Florian Engert)
2. Data Core (located at Johns Hopkins. Lead: Joshua Vogelstein)
3. Neuroengineering Core – provides support for engineering, machining and optimization of custom built equipment. (located at CBS. Lead: Aravinthan Samuel)
4. EM Core (located at Northwest Labs. Lead: Jeff Lichtman)
5. Live-Imaging Core (located at Biological Laboratories and Northwest Labs. Lead: Florian Engert)

Each of the Core facilities will provide administrative and technological support directly related to the four projects and thus be instrumental in accomplishing the three aims. Detailed descriptions and justifications of the core facilities are available in supplemental documents "Core facilities 1 – 5".

Significance and Innovation: An introductory note

The research plan we propose aims at a comprehensive multi-level understanding of how neural circuits generate behavior. Central to our approach is the idea, inspired by David Marr [7], that the brain implements *algorithms*, whether for sensory processing, decision making, or motor control, and that these algorithm can be inferred from careful observation of ethologically relevant behaviors. Once a particular algorithm is understood and delineated, we can interrogate its *neural implementation* by measuring from and manipulating the underlying neural circuits in the context of behavior. Results from such experiments can then be used to refine mechanistic circuit-level models of the brain. To be successful in coupling these different levels of analysis will require: (i) robust behaviors that can be described in terms of clearly delineated algorithms, (ii) a detailed understanding of the underlying circuits (anatomy and physiology), and (iii) the possibility to interrogate the function of those circuits in unbiased and rigorous ways.

We believe the larval zebrafish is uniquely suited for implementing such an approach. It has a few very robust and ethologically important behaviors that can be rigorously quantified and analyzed. It has a relatively simple nervous system that we can observe in its entirety while the animal is performing different behaviors [8]. Importantly, the field has matured, both in terms of available technology and a system-level understanding, to the point where unprecedented opportunities for doing integrative neuroscience have emerged [1]. For example, it is now possible to generate whole brain circuit models that are constrained by anatomy, functional imaging and behavior [1]. Currently, this is very difficult, if not impossible, to achieve in any other vertebrate. We believe our project takes full advantage of these developments by bringing together labs across the world with complementary expertise to work together on a well defined research plan. The behavioral component of our proposal (Project 2a) seeks to delineate the algorithms. The Atlas and the functional imaging component (Project 1 and Project 2b) will contribute to understanding the circuit level implementations of these algorithms, whereas the modeling component (Project 3) will synergize this information into a mechanistic understanding of how neural circuits underlie behavior. General principles underlying these algorithms and their neural implementations, that by definition hold true across model organisms, will be elucidated by close collaboration with expert laboratories working on rodents and Drosophila larvae (Project 4).

Progress report on the pilot project - the U01 proposal

Since this research proposal builds directly on the work accomplished within the framework of our U01 collaboration – and is thought as a continuation thereof - we briefly summarize it here.
The U01 proposal included two main goals: first, we proposed to generate a brain-scale circuit model of the optomotor response (OMR), an orienting behavior evoked by visual motion. To that end, we analyzed how whole-field motion is processed by a diverse set of neurons, which are distributed across multiple brain regions, and we demonstrated that these regions sequentially integrate eye- and direction-specific sensory streams and refine representations via interhemispheric inhibition to independently drive turning and forward swimming (Figure 1A). While these experiments revealed many neural response types throughout the brain, their respective connectivity remained unknown. In order to constrain possible connection weights, we used modeling to identify the dimensions of functional connectivity most critical for the behavior. Specifically, we used a dimensionality reduction analysis similar to PCA (principle component analysis) that operated on the high dimensional space of the synaptic weight matrix of our model, and we identified specific principle dimensions in which covariance between synaptic weights was high. Second, we established serial section electron microscopy as a technique that can combine whole brain functional imaging with whole-brain connectomics to further constrain and verify the specific connectivity matrices of such models in the future (Figure 1B,C)[2]. We thus revealed how distributed neurons collaborate to generate behavior and devised a paradigm for distilling functional circuit models from whole-brain data [1].

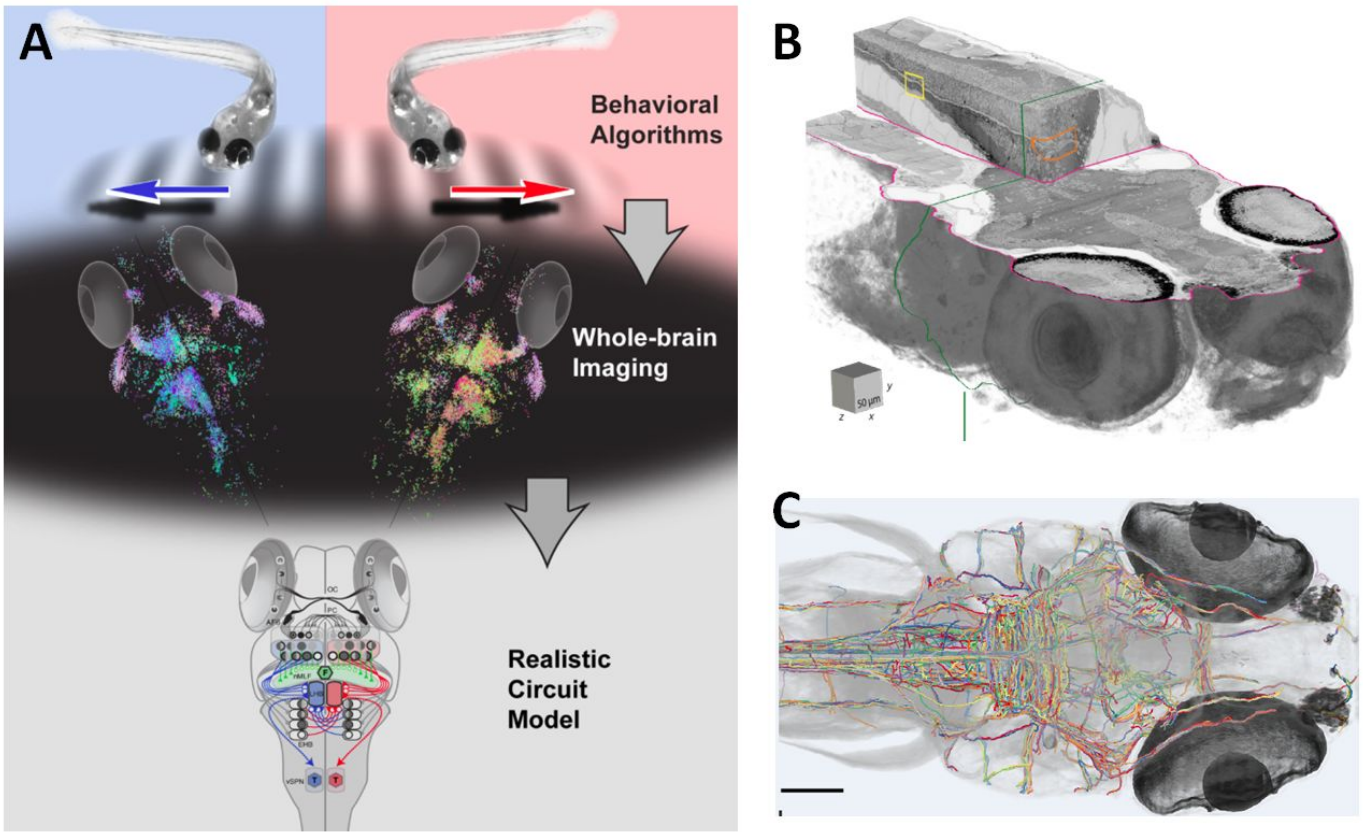


Figure 1: Summary of the U01 project. A) – whole-brain imaging and behavioral analysis combined with network modeling reveal key circuit elements contributing to a complex sensorimotor behavior in zebrafish larvae and provide a framework for building brain-level circuit models. **B)** – whole-brain serial section electron microscopy in the larval zebrafish. **C)** – reconstruction of all myelinated processes.

To summarize, the overall goal of the U01 proposal was to combine structure, function and theory in order to generate a realistic neural circuit model of the optomotor response in larval zebrafish and to establish serial section electron microscopy as a technique that can combine whole brain functional imaging with whole-brain connectomics to constrain and verify such models. Both aims were accomplished and resulted in high-level publications [1,2,9–14] that speak to the success of the collaboration. This gives us confidence that we can continue the project at a larger scale and with more ambitious aims.

Research Plan

In the following sections, we describe each aim of the research plan and highlight how the four projects ("Project 1 – 4") - as well as the core facilities - will be integrated into the overall plan. The central extension with respect to the U01 proposal is to add four behavioral paradigms (outlined in Figure 2) to the MVF (Aim 1), to study their interactions at the behavioral and circuit level (Aim 2), and to examine their modulation by internal states and context (Aim 3). We will also incorporate spontaneous swimming in the MVF as an additional behavioral paradigm. Spontaneous activity is of a general interest as it frequently displays universal character and functions [15] and it provides the background relative to which sensory motor responses should be measured.

Figure 2: A left turn executed in the context of all five behavioral paradigms. From left to right: **OMR, phototaxis, rheotaxis, escape, hunting**

Motivation for the choice of paradigms

The choice of these five behaviors is motivated by their prominence and importance in the larval zebrafish's repertoire. The survival of a fish larva depends, to a large degree, on three distinct behaviors: feeding [16–18], escape from predation [19–21], and maintenance of its location in potentially flowing waters. Hunting is associated with the first, and escape from looming objects with the second. Since these animals rely on vision to hunt and escape, attraction to well-illuminated areas (i.e., phototaxis) [22–24], is also adaptive. Further, both the OMR and rheotaxis serve to keep the fish stationary in a moving body of water and thus help it avoid being dragged into unfamiliar territory [3,4,25–27]. These five behaviors comprise a large fraction of the larva's behavioral repertoire and they all serve important, adaptive and evolutionarily conserved functions. Consequently, they constitute a meaningful basis for general circuit analysis (Aim 1) and are promising targets for the study of decision making under conflicting stimuli (Aim 2), since a larva must choose between these prominent behaviors under natural conditions. Furthermore, since most behaviors can be modulated by neurochemical internal states [28], it is likely that these five important and prominent examples are also amongst the targets of such state-dependent modulation (Aim 3). We therefore believe that these five behavioral paradigms not only allow for a detailed behavioral analysis and reconstruction of the underlying neural circuits (Aim 1), but also serve as the ideal basis for studying decision making under conflicting stimuli (Aim 2) and their dependence on internal states (Aim 3). Thus, the main motivation for selecting these five paradigms is their centrality to the observable behavioral repertoire, their evolutionary importance, and their accessibility. In addition, we have already made considerable strides in understanding these behaviors by developing quantitative assays [17,21,24,25,29–31], work that we will build on further in this proposal.

We note that although the behaviors that we will study in the fly larva, rat, and fish are broadly different, they are all tractable with the same approach in computational modeling. The stimulus-evoked and spontaneous behaviors that we will study in these animals can all be effectively reduced to underlying sequences of spontaneous and stimulus-driven movement patterns using modern techniques in behavioral reduction. General principles will be discerned in the overall pattern by which internal state adjusts neural and behavioral dynamics.

Aim 1 – Realistic circuit models for OMR, phototaxis, rheotaxis, escape and hunting

In the following, we describe these behaviors briefly and explain how Projects 1 and 2 will directly contribute to their integration into the MVF. The specific approach of how this information will be implemented in the modelling framework that comprises the MVF is described in Project 3.

OMR

When exposed to whole field moving visual stimuli, larval zebrafish will turn and swim to match the direction and speed of the stimulus, thus minimizing the motion on the fish's retina [3,4,26,31]. This is a robust algorithm for staying stationary in a moving body of water. Elucidating the precise behavioral algorithm as well as its neural implementation was a focus of our U01 project and the results have been published in Naumann, 2016 (see also Figure 1). The goal of Aim 1 is to extend and expand this study to four other behavioral paradigms.

Phototaxis

Many animals orient and move relative to the brightness of the environment, where they either seek an increase or decrease in light intensity. This behavior is known as positive or negative phototaxis, respectively, and zebrafish are known to exhibit strong positive phototaxis at the larval stage [22–24,29]. A larva can easily implement this behavior by comparing absolute light intensities between both eyes and executing directed tail flicks depending on the sign and value of the measured intensity difference [22]. Notably, there is an alternative way of performing taxis, often referred to as kinesis, where an animal has no access to differences in spatial intensity, but monitors absolute light changes over time and directs its behavioral output accordingly. Very similar to bacterial chemotaxis, a fish larva might swim straight when its locomotion leads to brightness increases and execute large angle turns when it leads to brightness decreases. We have shown that zebrafish larvae will readily execute both phototaxis and photokinesis [22,24], but for this proposal we will focus on spatial phototaxis alone, since it is conceptually and algorithmically easier to implement, and hence more readily modeled at the neuronal level.

Project 1 - Anatomy:

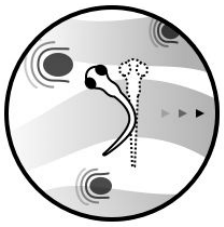
By exposing one or both eyes of head-fixed animals to varying light levels, we can identify the retino-recipient areas, also known as arborization fields (AFs), that are sensitive and selective to absolute light levels, by functional two-photon calcium imaging. These regions will be incorporated into the Z-Brain atlas, and will identify downstream postsynaptic candidate areas for calculating the inter-ocular light difference necessary for phototaxis. Such candidates can be verified by exploration of serial sectioned fish at the nanoscale with electron microscopy using the EM Core.

Prior studies have already identified the pretectum as an anatomically defined location where information from both eyes is integrated [1], making it a good candidate for an intraocular intensity difference calculator.

Project 2 – Behavior and imaging:

Closed loop behavioral assays allow us to update visual stimuli in relation to the position and gaze direction of freely swimming fish such that the images animals perceive are under complete experimental control. These assays are described in more detail in Project 2. They are ideally suited to quantify the intensity, frequency and probability of behavioral responses in larvae that are exposed to a constant difference in light intensity delivered to the two eyes. The very same stimuli can be delivered to animals in a head fixed, but tail free, preparation or to paralyzed fish swimming in a virtual environment [8]. Concurrent brain-wide calcium imaging will then allow to test several hypotheses: 1) Is the pretectum, which was shown to be the first location of binocular integration in the OMR study [1], also the stage of binocular processing in the context of phototaxis? 2) Are the same neuronal response types involved? 3) Is the same arborization field (AF) responsible for relaying information from each eye? Or are more than one and different AFs involved? Once these questions are resolved, we can further determine whether and how the processed information about inter-eye intensity differences is communicated to the hindbrain centers that are known to generate appropriate motor outputs for the OMR [4,22,32].

Rheotaxis



Aquatic animals often adjust their own movement to compensate for being dragged by the flow of the surrounding water. These flow-induced displacements can most easily be detected as apparent visual whole-field motion with respect to the animal's frame of reference. In order to compensate and swim against the flow, the most effective strategy is to swim along with this apparent whole-field motion and thereby stabilize the retinal image. Such behaviors can be categorized as reflexes, such as the OMR (discussed above) and the optokinetic reflex (OKR) [33]. Nevertheless, many aquatic animals consistently orient and swim against oncoming water currents (a behavior known as rheotaxis) even in the absence of visual cues. We recently demonstrated that larval zebrafish perform rheotaxis by using local flow velocity gradients as navigational cues, and have proposed a novel algorithm based on such local velocity gradients that fully explains the details of the behavior [25]. Specifically, we showed that fish use their mechanosensory lateral line to sense the curl (or vorticity) of the local velocity vector field, and that this curl serves as the central variable to be measured for effective rheotaxis. These results revealed an elegant navigational strategy based on the sensing of flow velocity gradients and provide a comprehensive behavioral algorithm, also applicable for robotic design, which generalizes to a wide range of animal behaviors in moving fluids.

Project 1 - Anatomy:

The sensory receptor cells providing the main input for rheotactic behavior in the dark are the mechanosensory hair cells of the neuromasts in the lateral line. The team of López-Schier has made significant advances in categorizing and cataloguing these sensory cells, including their afferent and efferent neuronal populations [34,35]. These anatomical descriptions will be augmented by serial electron microscopy reconstructions that will provide valuable additions to the Z-Brain atlas, which will be further enriched with anatomical details related to the nuclei relaying the somatosensory information - the Medial Octavolateralis and the Torus Semicircularis - to higher order processing centers and the motor output units in the hindbrain.

Project 2 – Behavior and imaging:

Details of the behavioral algorithm are outlined in Oteiza et al., 2017 and Project 2, 3. To enable brain-wide calcium imaging in the context of rheotaxis, a fictive swimming set-up will be constructed that allows the delivery of gentle and directional water-currents targeted to the lateral line of the fish. Such fictive swimming set-ups are already in routine operation (See Project 2) and are readily adapted to the rheotactic paradigm. Once this fictive swim set-up is made compatible with whole-brain imaging, the hypothesized circuitry (described in Project 3 in detail) can be identified by tracing the predicted signals inward from the neuromasts.

Escape

A larval zebrafish exposed to a quickly expanding visual stimulus will execute, like many other animals, a rapid, and often directional, escape maneuver to avoid collision with the potentially predatory object represented by such a sensory signal [19,36,37]. In collaboration with Filippo Del Bene's group we have recently quantified this behavior and described the precise stimulus statistics necessary for triggering it. We also made inroads into dissecting the underlying neural circuitry in the larval tectum and the hindbrain [21].

Project 1 - Anatomy:

The Del Bene team has in recent years added to the catalogue of cell types with specific response properties and projection patterns [38–41]. These cell types will serve as likely candidate neurons for the specific processing necessary to distinguish an expanding stimulus from other visual cues. Additional anatomical information is also needed and will be added to the atlas, with respect to the Mauthner cells (a pair of big and easily identifiable hindbrain neurons known to be responsible for very fast escape reflexes) and its three homologues. It is clear that these four pairs of neurons play a necessary role in mediating looming evoked escapes, but their precise function and connectivity remains to be fully elucidated. The EM Core will provide the exquisite synaptic level resolution of the input and output of these neurons.

Project 2 – Behavior and imaging:

The proposed experiments will be structured around paralyzed fish swimming in a virtual environment (see "Project 2") where they will encounter unpredicted looming objects of various speeds and sizes. Brain wide calcium imaging concurrent with spontaneous behavior as well as looming evoked escapes will allow us to identify the relevant brain areas and circuits. Pilot studies to that end have been published already by several of the team members [17,21,38].

Hunting

During hunting, larval zebrafish first recognize specific visual objects as prey and then perform a sequence of goal-directed turns and swims to approach their target before finally striking at it. Individual neurons in the visual pathways have been proposed to function as 'feature detectors'. Such neurons are selectively excited by specific spatio-temporal patterns within the visual scene and include neurons that are tuned to visual features that define key stimuli [42–44]. Despite the major contributions of such studies, it has been difficult to define the complete neural circuit controlling hunting behaviors. For example, we don't yet understand how the activities of individual neurons, as well as the dynamic spatiotemporal patterns of activity spanning multiple brain regions, mediate the sensorimotor transformations that convert visual input to motor output. We have made first inroads into uncovering the circuitry underlying prey capture by establishing a head-fixed assay that allows for the study of the initial phases of the hunting sequence – object acquisition and tracking [17]. In the context of this study the optic tectum was identified as a critical nucleus for prey capture and several neuronal response types were found that seem to play a critical role [18].

Project 1 - Anatomy:

The extended networks of periventricular tectal neurons [18], together with the arborization fields of retinal ganglion axons that provide the prefiltered input [45] will be added to the atlas. In addition, work from the Del Bene lab has revealed the involvement of a particular class of size-selective inhibitory neurons [38] and intra-tectal connecting neurons (Del Bene, unpublished observations). This information will also be integrated into the atlas, together with the activity propagation to pre-motor areas in the hindbrain (see "Project 3"). The EM Core can add information about specific connectivity of these neurons through sparse reconstruction of functionally identified circuits.

Project 2 – Behavior and imaging:

Behavior: we have started pilot experiments that significantly expand our ability to monitor and analyze hunting behavior. To that end, we designed and built a set-up where a high-resolution, high-speed camera tracks and follows a freely swimming fish larva that navigates in an arena that spans several feet in diameter. This apparatus, termed the BEAST, allows for detailed Behavioral Evaluation Across Space and Time, not only of the fish but also of all the prey objects (usually paramecia) surrounding the fish. The resulting data sets are essential for determining the algorithms and mechanisms that allow a larva to first select and then continuously attend to an individual paramecium when confronted with hundreds of distractors (see "Project 2" for details). The data sets collected by the BEAST will allow us to extract the precise algorithms and boundary conditions that govern hunting behavior in fish larvae (see "Project 3").

Imaging: Preliminary studies using head fixed preparations have already shed light on the sensory circuitry underlying the initial filtering and processing of prey-like stimuli [18]. These studies will be expanded by upgrading the BEAST to become compatible with wide-field fluorescence imaging, by extending the fictive behavioral paradigm to complete fictive immersion (see "Project 2") and by extending volumetric imaging from the tectum to the entire brain.

Project 3 - Modeling of all five behaviors:

The specific approach of how this information will be implemented in the modelling framework that comprises the MVF is described in Project 3. For all five behaviors, the modelling approach will follow the same general principles and approach as described in our U01 summary, as well as in Naumann et al., 2016 for the OMR. All five behaviors will be integrated into a single, unified simulation framework.

Potential pitfalls, shortcomings and outlook - Aim 1

The biggest concern with respect to all five paradigms is that their successful fictive implementation might not work. For example, tethered larval zebrafish will readily execute the initial phases of prey acquisition and tracking, but they fail to execute the complete sequence including prey pursuit and strike. To address these shortcomings we will complement the tethered brain-wide calcium imaging experiments with two complementary functional imaging approaches that are compatible with freely swimming animals. The first has been recently established by the Schier/Engert group and is already routinely used in a variety of projects [6]. Here we take advantage of the fact that intracellular ERK (Extracellular signal–regulated kinase) is phosphorylated upon activity dependent calcium entry into a neuron and can be visualized, posthoc, with selective antibodies against pERK and tERK, the phosphorylated and non-phosphorylated forms respectively. The second approach, which is still under development, entails upgrading the BEAST with wide-field fluorescence capabilities. This is a straightforward technical challenge, where significant progress has already been made with the help of the existing CBS engineering core (see "Engineering Core").

Furthermore, the existing Z-Brain does not include any detailed information on spinal cord circuitry, or the retina which is the main sensory input for our behavioral paradigms. Both of which are also conspicuously missing in the circuit model presented in Naumann et al., 2016 and the U01 project. Since the spinal cord serves as the critical link between the brain and behavioral output, we propose to add it as a specific module to the atlas (see "Project 1") as well as the MSV (see "Project 3"). The essential knowledge and expertise from the Wyart and McLean laboratories (spinal circuitry) as well as the Douglass group (descending modulation) will be necessary to complete the incorporation of spinal cord anatomy, physiology and circuitry to the atlas. Retinal circuitry, physiology and modeling will be implemented with the dedicated support and help from John Dowling, a world renowned expert on the retina and a member of our extended fish team. Altogether, we are optimistic that we can construct a single realistic circuit model that can explain a variety of different sensorimotor transformations.

Aim 2 –Decision making in the presence of conflicting and multimodal stimuli

Under natural conditions, animals usually get bombarded with a multitude of sensory stimuli that enter the brain via a variety of receptor types representing different modalities. It is then the task of the brain to filter this plethora of information such that the most appropriate action is selected given the momentary context. How this process of filtering and selection is executed in a brain interacting with the world in real life situations is not yet clear. One reason for this lack of understanding is that most experiments are focused on specific sensorimotor tasks where the experimental context is optimized for easy interpretability and explicitly made void of any distractors.

We propose here to investigate how animals respond to conflicting and possibly opposing stimuli at three levels. First, we will test at the behavioral level what happens if larval zebrafish are exposed to a variety of conflicting stimuli that evoke predictable behaviors when presented in isolation. Second, we will perform whole-brain calcium imaging in animals that fictively navigate a virtual environment where they are exposed to a similar array of conflicting stimuli. Third, we will refine and expand the MVF such that behavioral, as well as imaging results under these conditions are reproduced and accounted for by the circuit model.

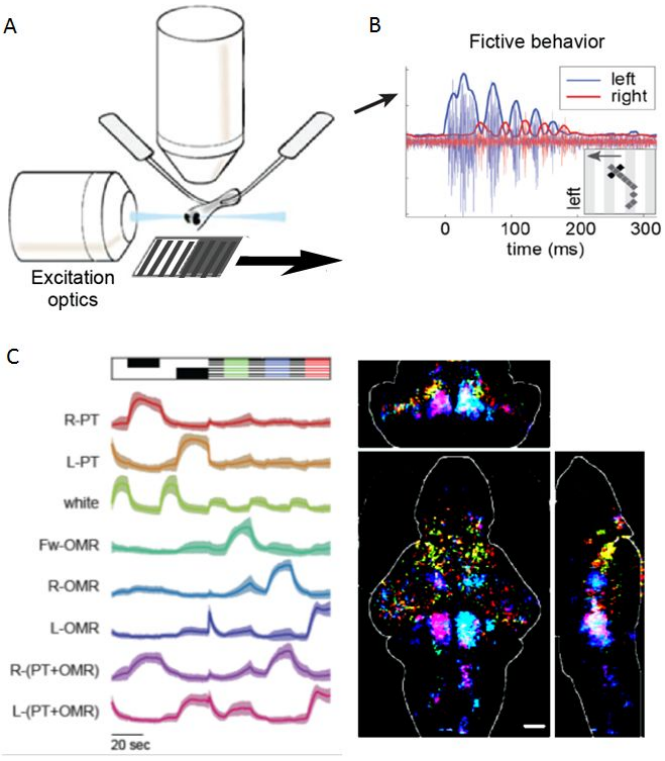


Figure 3 - brain wide imaging under conflicting stimuli conditions: A – light sheet imaging in a fish fictively responding to a conflicting stimulus. OMR grating moving to the right but the field of view is brighter on the left. B – example of a fictive turn to the left. C – examples of eight different neuronal clusters responding to different aspects of the stimuli. Top left indicates the stimuli: pure phototaxis in black and white; gratings moving forward, right and left shown in green, blue and red respectively.

As an example, we discuss a specific case where a larval zebrafish has to decide between two conflicting behaviors – phototaxis and OMR. In this paradigm, the larva is exposed to whole-field gratings moving to the left, but the overall brightness of the stimulus is higher on the right (see schematic in Figure 3A). Both stimulus intensities can be adjusted gradually: the OMR grating by increasing its contrast, and the left-right brightness difference by changing its absolute and relative magnitude. Within a single trial in a closed loop freely swimming assay, we can thus change the conditions from all-OMR to all-phototaxis and quantify the precise settings of the stimuli. The same experiment can be undertaken with a paralyzed fish fictively swimming in response to the same stimulus conditions (schematized in Figure 3A, B). Concurrent brain-wide imaging and analysis of responsive neuronal ensembles has already been established in the Ahrens and Engert labs. We can readily expose fish to a variety of phototactic and OMR related stimuli in sequence (Figure 3C), though concurrent delivery of these stimuli has yet to be tested. This will enable the tracing of information pathways for each individual stimulus set and the identification of the brain areas and local micro-circuits responsible for switching the behavioral response from one stimulus to the other.

In the first five years of the research plan, we will explore the behavioral consequences of sequential and simultaneous delivery of all five stimulus sets related to our behavioral paradigms. The resulting data will be used to constrain the part

of the circuit model within the MVF that has been established for the individual behaviors as outlined in Aim 1. In the case of the OMR vs. phototaxis paradigm outlined in Figure 3, we will focus initially on brain areas and target cells – possibly located in the pretectum – that respond to the OMR and phototactic stimuli specifically or in combination. These cell types may be distributed over several areas and may change their response modes gradually, or they could be specialized to a specific nucleus and switch their response modes in a single step.
In principle, we can test all possible combinations of the five associated stimulus sets – OMR, phototaxis, rheotaxis, escape and hunting – since each stimulus evokes a clearly predictable turning behavior to the right or the left. Of all ten possible pairwise combinations (4+3+2+1) we propose to start with the candidates most likely to provide clear and easily interpretable results, based on their ready implementation in head-fixed or fictive assays (see Project 2 for details)..
Finally, we propose to match not just two but three or more stimulus sets against each other in order to more accurately mimic natural conditions, where many relevant stimuli are likely to occur simultaneously. Here an almost endless variety of combinations is possible, but we will first select the three stimulus sets where we already have the largest set of preliminary data at the behavioral and algorithmic levels, and therefore the highest confidence of success. These three paradigms are OMR, phototaxis and rheotaxis. In order to implement stimuli relevant for rheotaxis, we will add the ability to generate local flow gradients to the setup described in Figure 3. We can then match OMR, phototactic and rheotactic cues systematically against each other. As explained in Aim 1, local water-flow gradients will indicate the presence of a current, and a gradient increase or decrease following a swim bout allows the animal to extract the direction of the flow [25].
Several outcomes are possible from these experiments at the behavioral as well as the brain-wide imaging level: the added third stimulus might add linearly to the existing other two, or hypo- or hyper-linear additions might occur. An extreme alternative to linear addition would be the case of an all or none dominance of an individual stimulus set. A looming object, for example, might override all other simultaneously presented stimuli and dominate the behavioral output. Experiments such as this will allow us to ask questions that go beyond the relatively simple case of just two conflicting stimuli.
Any of these possible outcomes will be implemented in the MVF by supplementing the circuit model with the necessary non-linearities - at the synaptic level or the amount of excitability of individual neurons (See Project 3).

Potential Pitfalls - Aim 2
The success of these experiments will rely, amongst other things, on the robustness of the behavior under tethered or paralyzed conditions. We have preliminary data showing that this works well for OMR and phototaxis, but when tethered, larvae fail to perform the final stages of the hunting sequence (pursuit and capture). Rheotaxis and escape have not been systematically tested under tethered conditions and their inclusion in the conflict/decision related experiments will depend on the outcome of the proposed pilot studies in Aim 1. Our previous success successfully implementing fictive behaviors [8,15,46,47] makes us hopeful that we will also succeed with rheotaxis, escape and hunting. However, if these efforts should fail we will 1) focus on the already proven and established paradigms, 2) we will use the information extracted from freely swimming imaging technologie (pERK and fBEAST, see Project 2), and 3) we will analyse the activity in sensory areas that are known to be little affected by immobilization.

Aim 3 – Modulation of sensory-motor transformation by internal states

All animal behavior is controlled and explained not only by sensorimotor transformations, but also by the modulatory influence of “internal states”. Within the context of this proposal, we propose the following operational definition of an internal state:
An internal state reflects endocrine and neurochemical factors that influence information processing in neural circuits (Fig 4) and change the release probability of behavioral outputs. Internal states are modulated by external events (e.g. presence of predators, prey or mates) and environmental conditions (e.g. temperature, lack of food, etc).
Examples of internals states that we are able to experimentally manipulate are hunger (induced by food deprivation), stress (induced by pain), and loneliness (induced by social isolation). We note that this definition of internal state explicitly excludes experience-dependent circuit plasticity. As such, we will take care to use animals that are naive with respect to the behavior that is modulated.
The neural mechanisms that alter internal states operate at a far slower time-scale than those involved in the sensorimotor transformations described in Aims 1 and 2, which rely on millisecond precision neural processes. Importantly, these different processes interact, with slow changes in the internal state of the animal affecting information processing in fast sensorimotor circuits (green arrows in Figure 4), which underlie behaviors such as rheotaxis, phototaxis, escape or

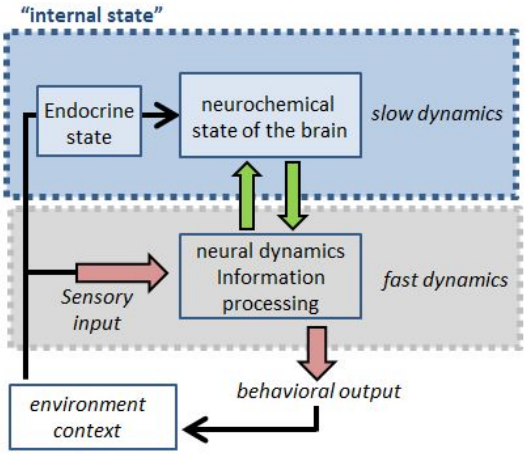


Figure 4 – Schematic illustrating how slow, modulatory internal state circuits (blue) interact with fast sensory motor networks (grey). Red arrows indicate interactions of the outside world with the fast networks, green arrows the mutual influence of the two systems on each other.

hunting. Explicit examples of such interactions are a preference for hunting behavior over escape in hungry fish [20,48], increased sensitivity to noxious stimuli in stressed animals [49] and increased arousal thresholds in sleep deprived animals [50]. Notably, all of this applies equally to flies, fish and rats and, as such, offers fertile ground for cross-species comparisons. The first phase of the project, years one to five, will focus primarily on the characterization, control and neural basis of internal states associated with stress, hunger, social isolation – as well as several intermediate internal states such as hunt/explore (see "Project 2/3"), swim-gain [46] and turning bias [15]. Two specific cases of slowly changing internal states and their modulation, related to stress and hunger, are described in more detail in "Project 2". There we describe pilot data that have been collected in collaboration with the Douglass and Kunes groups that isolate two systems in the hypothalamus, including oxytocin and serotonin-positive neuronal populations, that modulate internal states related to stress and hunger in interesting ways, including the likelihood to engage in hunting or to respond to noxious stimuli.

For all of these experiments, it is essential to quantitatively determine the internal state of the animal. To do this we will use three independent methods: (1) control of the environmental context (e.g. noxious heat for stress, food deprivation for hunger), (2) behavioral readout (increased frequency of escape behavior for stress, increased hunting activity and prey ingestion for hunger), (3) recording of related neurochemical state (increased activity of oxytocin neurons during stress; modulation of serotonergic activity in the caudal hypothalamus for hunger, see "Project 2"). In the second phase of the project, years six to ten, we will shift the focus on analysing the modulation of the fast sensory-motor transformations described in Aims 1 and 2 by various internal state changes. We will also harness the expertise of the whole team in zebrafish optogenetics to perturb and modulate the neurochemical state of the brain directly. The resulting changes in sensory motor transformations can then be analyzed at the neural network level (through brain wide imaging) as well as at the level of behavioral output (through detailed and quantitative behavioral analyses).

Our work towards Aim 3 is distributed over the four projects as follows:

Project 1 – Atlas:

As a first step we propose to catalogue, map and characterize the modulatory systems thought to be involved and responsible for internal states. This expansion of the Z-Brain atlas will be an ongoing process that requires the continuous and synergistic effort of the whole team. Specifically, the viral tracing technology made possible by the dedicated support from the Cepko group allows us to add essential data about projection patterns of genetically identified cell groups to this database [51–54]. Furthermore, new transgenic technology from the Halpern group will add significantly to our ability to label and perturb these populations in live animals [55–58]. A more detailed description of all neural cell types, brain areas and modulatory neurotransmitter systems is included in Project 1. To supplement the existing preliminary atlas (Z-Brain) will ultimately require the concerted effort of the international zebrafish community and we believe that the framework of the U19 award provides us with a possibility to establish the necessary infrastructure for such an ambitious enterprise.

Project 2 – Behavior and Imaging:

We have successfully employed a variety of methods to change the internal state of larval zebrafish. Specific examples are changes of ambient heat, social isolation, chronic activation of nociceptors and food deprivation. In addition to these long term modulations of internal states, we also have observed and induced short-term modulation of behaviors in the context of hunting (search vs feeding), motor gain learning (consequences on swim vigor depending on feedback gain in previous swim bouts [46]), and exploratory bias (a bias to string together swim bouts into a certain turn direction) [15]. In addition, we have put in place high throughput technologies with which to screen thousands of animals for the behavioral consequences of various modulatory manipulations [30] (see Project 2).

Project 3 – Modeling and Theory:

The slow dynamics of internal brain states (schematically outlined in Figure 4) will be modeled as Markov transitions, where we will explore how internal states modulate not only gains and biases of neuronal mean responses but also response variability (noise) and its spatial correlations. This framework, explained in more detail in Project 3, will be used to model quantitatively brain state changes related to social loneliness and hunger, both conditions that are subject to experimental manipulations in larval zebrafish. We will use measured activity changes as a brain state readout to: (1)

identify internal brain states (for instance hunger or social stress may comprise more than one state) and their transitions, using clustering and Hidden Markov Model methods; (2) model the functional neuromodulatory circuits; (3) identify the specific sensorimotor circuits that exhibit state dependent modulations of their fast dynamics; (4) build minimal models incorporating modulation of neuronal/synaptic gains and/or response, sufficient to explain the observed changes in behavior and neuronal activities across the larval brain; and (5) refine these models based on further experiments on the same systems. All neuromodulatory circuits studied will be incrementally merged into MVF (see Project 3) and will be validated by the following means: 1) EM assisted sparse circuit tracing of functionally identified neuronal types (EM core). 2) viral assisted circuit tracing 3) targeted neuronal laser ablation and chemical silencing 4) targeted activation through optogenetic and chemical approaches. The latter will be achieved with help of the Douglass, Del Bene and Halpern groups, who will generate Gal4 and QF reporter fish lines for specific neurotransmitter/neuropetide populations, adding to the existing repertoire of oxytocin, hypocretin, Substance P and Serotonin-specific transgenic lines (see Project 1 and 2). During fictive behavior, selected neuronal populations can then be activated by crossing reporter lines with UAS or QUAS-driven light-gated ion channels such as ChR2 and NpHR. These, and other modern neuronal silencing or activation techniques [59–61], whose application is greatly facilitated by the CRISPR-Cas system [62], will be valuable tools to interrogate circuits specifically and help to validate and constrain the role particular neuromodulatory systems play in contributing to the MVF.

Project 4 – Other Model Systems - General Principles:

Modulation of sensory motor processes by internal state is a general feature that all animals have in common. Thus, we will compare the specific cell types and circuits elements already discovered in *Drosophila* larvae (Project 4), as well as the state transitions that become apparent from detailed analyses of the much richer behavioral repertoire of rats (Project 4) to the emerging principles we hope to uncover in the zebrafish within the framework of this proposal. Such common principles may include the specific rules by which slow neuromodulators can change the dynamic properties of fast sensorimotor circuits.

Potential Pitfalls - Aim 3

The success of these experiments will rely, like Aim 1 and Aim 2, on the robustness of the behavior under tethered or paralyzed conditions. Here the primary concern is that head embedding (or paralysis) in itself is likely to change the internal state of the animal. However, our past experience with successfully implementing fictive behaviors [8,15,46] and their quantitative comparison with those of freely swimming fish [47], indicates that animals will adapt readily to the tethered conditions and that significant induced internal changes will occur under both conditions given the appropriate environmental context. In addition, we will use the information extracted from freely swimming imaging technologies (pERK and fBEAST), to cross-validate and confirm the data and findings collected from experiments performed on tethered fish.

Team structure - Philosophy - Timeline

There has been a lively debate in the past years about the respective advantages of big, industrial scale science in the theme of the Genome Project, the Allen Institute or the Human Brain Project versus the small to medium scale approach favored by many individual laboratories led by sole PIs. We will label these two approaches the “corporate” and the “cottage industry” way of doing science, respectively. Advantages of the corporate approach are an obvious increase in efficiency and productivity if clear goals and milestones are in place [63]. However, for many avenues in neuroscience the corporate approach is difficult to implement because clear goals, business plans and milestones are hard to define. The flexible structure of the “cottage industry” approach, on the other hand, is characterized by quick response times, which permits dynamic adjustment to new ideas, new technologies and alternative approaches. One significant caveat of this approach is that it is often compromised by several sources of inefficiencies: individual laboratories keep re-inventing the wheel in terms of technological approaches, they often unknowingly work on very similar questions and they often actively compete - which leads to a deliberate containment of information and technology and often generates undue haste in getting results to publication. Our proposal capitalizes on the positive aspects of both approaches: large-scale open collaborative systematic data collection and infrastructure building, combined with focused investigator- and hypothesis-driven research. The core challenge for the large team we propose will be to provide a framework and infrastructure that ensures an efficient and synergistic collaboration of all team members. Our main goal is to bring together a large fraction of leading scientists working on zebrafish neuroscience within the US and Europe in a well-organized and, importantly, friendly and collaborative framework, one that emphasizes synergy and avoids

redundancies and multiplication of efforts. Specifically, outright competition, which frequently promotes secrecy, undue haste and discord, will be avoided at all costs. The hope is that we can avoid most of these issues by promoting an atmosphere of open collaboration, support and resource sharing that can be guaranteed by integrating all efforts within the framework of the U19 structure.

The field of zebrafish neuroscience is relatively young but has recently blossomed into a promising active and synergistic community. We will use the opportunity of the U19 framework to support and promote these interactions and synchronize and coordinate the efforts across all groups. Figure 5 illustrates how all proposed team members fit into the overall proposal. Everyone will contribute complementary skill sets which will help accelerate the process significantly and add components that the core team otherwise would not be able to address. Finally, we note that the four scientific cores we propose to establish, Data, Engineering, EM and Live-Imaging, need both the expertise and the financial backing that the combined team is ideally positioned to provide. Once established, they will provide valuable and large-scale resources to the neuroscience community, but their establishment is best motivated through a team whose size needs to exceed a certain critical mass to justify the expense and effort. We believe that with our proposal we strike the ideal balance between a large enough number of individual laboratories to justify such an endeavor and an overall size that is still manageable and lean enough to stay flexible and to minimize the significant inertia that necessarily accompanies the corporate approach.

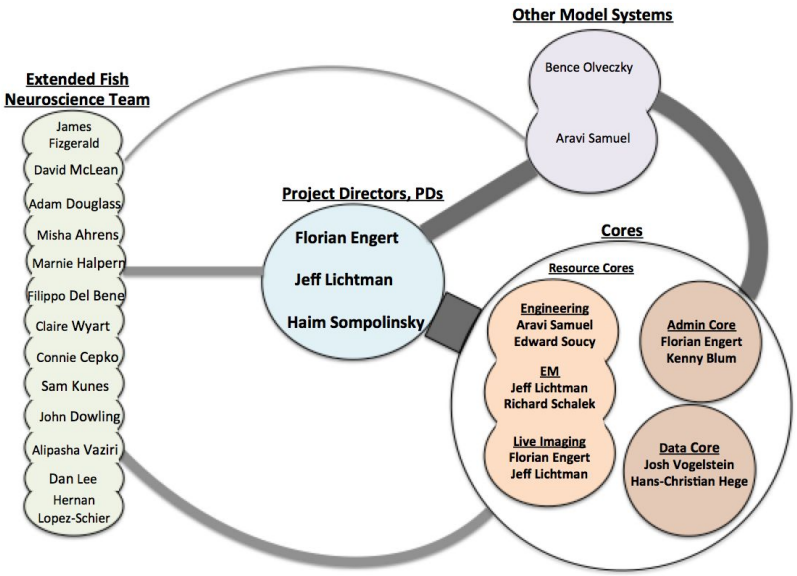


Figure 5: The Team and the Cores. Expected relations between the individual elements are encoded by thickness and intensity of connectors.

We acknowledge that this hybrid approach between corporate and cottage is novel and comes with a certain risk, but the reward would be significant and the U19 framework provides a unique opportunity to go forth and test such an exciting and promising approach.

Timeline:

We believe that the proposed experiments within the overall research project can be accomplished within a projected timeframe of 5 years and we have outlined the anticipated timeline for each of the Aims and sub-Aims below.

		Year 1	Year 2	Year 3	Year 4	Year 5	Year 6-10
Aim 1	Development of Fictive Assay Imaging Experiments in Fictive Assay pERK Imaging in Freely Swimming Assay Modeling (MVF)						
Aim 2	Decision Making and Conflict Resolution Modeling (MVF)						
Aim 3	Characterization of Internal States Analysis of Effect of Internal States on Aim1 and 2 Modeling (MVF)						

Project Timeline: Aims 1, 2, and 3 (and sub-aims) are color coded in darker shades of blue, green and purple respectively.

It is clear that all of the Aims are open ended, however, we are confident that specific hypotheses can be successfully confirmed or rejected within this time window and that significant progress can be made at adding to our understanding of how neural circuits encode sensory information, of how they control behaviors under a variety of conditions and of how they are modulated by ethologically relevant internal neurochemical states. Independently of the success for the individual aims, the creation of the MVF as a modeling and simulation platform, as well as the generation and curation of the Z-Brain Atlas, are guaranteed to provide essential and extremely useful contributions to the neuroscience community.

Vertebrate Animal Section-Zebrafish

Performance Site(s):
All vertebrate animal work will be performed at the facilities of Harvard University, Faculty of Arts & Sciences (HU/FAS). The HU/FAS animal care and use program maintains full AAALAC accreditation, is assured with OLAW (A3593-01), and is currently registered with the USDA.

1. Description of the proposed use of animals

Behavioral assay
IACUC approved protocol 23-03
500 zebrafish larvae (5dpf) will be used for somato-sensory stimulation and behavioral testing; the procedure is straightforward involves no restraint or surgery. Briefly, freely swimming individual animals are observed by a camera and subjected to moving water currents of certain speed, shape and orientation. After a variable time in this behavioral chamber animals are transferred back into the holding tanks.

Two-photon imaging and head fixed behavioral assays
IACUC approved protocol 22-04
Zebrafish larvae between 3 dpf and 6dpf in age will be immersed into a drop of solution containing 1mg/ml alpha-bungarotoxin for five to ten minutes. Standard safety procedures for handling this toxic substance are followed (safety goggles, facemask, double gloves and no sharp objects). Care is taken not to exceed a total amount of 1mg of the substance at any given time. LD50 is 0.15 mg/kg. Thus a dangerous dose for a lightweight human would be ~ 10mg injected intraperitoneally. This treatment paralyzes the larva and all movement stops while heartbeat continues unchanged. Larva up to 20 days post fertilization do not yet have functioning gills and oxygen is supplied entirely by diffusion through the skin. It is therefore not necessary to supply oxygenated water in any particular form. Larvae that recover from paralysis after several hours show no change in their behavior when compared to non-treated animals. After induction of paralysis, larva are transferred into normal fish water for imaging by two-photon microscopy. To this end fish are suspended in mid-water by three large bore glass suction pipettes. Very mild negative pressure is applied to the pipettes such that they attach to the intact skin and the animal can be moved into position for imaging. Two of these glass pipettes do double duty and record motorneuron activity through the skin. The procedure is entirely non-invasive. To probe sensory processing larvae are either exposed to different somatosensory stimulation by moving water over the skin of the fish. The water-flow is delivered through pipettes positioned in the vicinity of the fish.
Fictive struggles stop after the first couple of minutes and animals respond well and predictably to stimuli that elicit routine swim turns. When fictive struggles are recorded for more than five minutes the experiments is terminated and the animal euthanised.
Each fish may be imaged for up to 6 hours. The primary target neurons will be stained with genetically encoded fluorescent proteins. Anesthesia isn't possible under these conditions because the drug will interfere with neuronal processing.

After the imaging sessions fish will be removed from the microscope and returned to standard Petri Dishes in BL-2075. On subsequent days they might be subjected to repeated (up to four) imaging sessions on up to four different days. That is at most one imaging session per day.
Ultimately fish will be euthanized by immersion in 2% of buffered MS222 and, sometimes, fixed for imaging by electron microscopy.

2. Justification:
We study neural processing of information, this needs to be done in a living animal. Very little insight can be gleaned from cell cultures or slices and the zebrafish embryo is about the simplest and least evolved vertebrate existent. Furthermore, with its wide availability of genetic markers and its small translucent brain it is ideally suited for functional calcium imaging studies.
Species for all described experiments are zebrafish larvae (Danio rerio) between 3 and 6 days post fertilization. Sex cannot be determined at this stage of development. For imaging and behavioral experiments we will use 500 larvae per year. We plan on performing up to 10 experiments per week with one fish per experiment. To establish the different technologies about 400 experiments are necessary (100 each). To get significance we'll need at least 25 for each group which make up the remaining 100.

3. Provisions to minimize distress, discomfort, pain and injury:
For all potentially painful procedures animals are anaesthetized before the start of the experiment with 0.02% MS222. For the terminal procedures, they are euthanized by immersion in a lethal dose of 2% MS222.

Veterinary care
Veterinary care is not available to monitor the fertilized eggs and fish embryos in the lab incubators. The main Biolabs fish facility in the basement where older fish are kept is inspected regularly and has a constant staff of three technicians. This facility is part of the Harvard University/Faculty of Arts & Sciences veterinary care program which is overseen by an attending veterinarian and supported by a veterinary pathologist, four clinical veterinarians, and three veterinary technicians. Veterinary care is available 24/7 via routine rounds and a rotating on-call schedule. Veterinary staff rounds are performed 2x week with additional exams as needed through all animal holding areas. Procedures in progress at the time of rounds are observed. The veterinarians work directly with the animal care staff on programs designed to reduce the prevalence of infectious disease, the monitoring of animal health, and the diagnosis and treatment of illness and disease. The veterinary staff reserves the right to intervene in all cases in which animals are experiencing unalleviated pain or distress that has not been justified in the protocol as necessary to accomplish scientific objectives and for which provisions for palliative care have not been provided.

4. Euthanasia:
For terminal experiments animals are euthanized via immersion in 2% MS222. When the animals become too old for imaging (exit the larval stage) or are determined to be ill or injured, they will be euthanized via immersion in 2% MS222. These methods are consistent with the recommendations of the 2007 AVMA Euthanasia Guidelines.

Vertebrate Animals-Rats

IACUC approved protocol 29-15
Performance site
All vertebrate animal work will be performed at the facilities of Harvard University, Faculty of Arts & Sciences (HU/FAS). The HU/FAS animal care and use program maintains full AAALAC accreditation, is assured with OLAW (A3593-01), and is currently registered with the USDA.

Proposed use of animals
Approximately 60-70 rats (Long Evans 150 grams) will be used for the proposed experiments. The majority of these will undergo craniotomy under 2% isoflurane anesthesia.

Justification for the use of animals
We are using rodents as model organisms for understanding how internal state changes affect neural dynamics and behavior. Rats have been studied for a long time in both laboratory and natural settings, and they have history of being used for behavioral studies. In initial studies involving our paradigm, we have been able to collect consistent and reliable data, motivating their continued use them as a model species for this grant. The PI has prior experience in the handling of rats and their use in comparable experimental situations. All procedures will be designed to minimize pain and discomfort for animal subjects.

Veterinary care

The Harvard University/Faculty of Arts & Sciences veterinary care program is overseen by an attending veterinarian and supported by a veterinary pathologist, four clinical veterinarians, and three veterinary technicians. Veterinary care is available 24/7 via routine rounds and a rotating on-call schedule. Veterinary staff rounds are performed two times a week with additional exams as needed through all animal holding areas. Procedures in progress at the time of rounds are observed and cage-based medical records are reviewed to assure adequate and timely administration of pain relieving drugs and palliative provisions. The veterinarians work directly with the animal care staff on programs designed to reduce the prevalence of infectious disease, the monitoring of animal health, and the diagnosis and treatment of illness and disease. The veterinary staff reserves the right to intervene in all cases in which animals are experiencing unalleviated pain or distress that has not been justified in the protocol as necessary to accomplish scientific objectives and for which provisions for palliative care have not been provided. All personnel involved in animal handling and surgical procedures will acquire certification through institutionally approved animal care training courses for rodents including the completion of AALAS Learning Library training modules.

Procedures to limit pain and discomfort

For the craniotomies required for our experiments rats will undergo a skin incision under 1-2 % isoflurane anesthesia; body temperature will be maintained at 37 °C using a feedback-controlled heat pad (Harvard Apparatus). Throughout the procedure, depth of anesthesia is checked by monitoring of tail pinch response, whisking, breathing rate, and eye-and toe reflexes. Heart rate and partial oxygen pressure will be monitored to ensure the animal's condition. Animals are placed in a stereotaxic apparatus with the head secured by non-puncturing ear bars. Surgery involves longitudinal incision of the scalp, which is retracted to expose the underlying skull. Craniotomies over the parts of the brain we will record from are then made using a drill. The probes are placed into the brain and the craniotomy is covered by Kwik-Cast sealant, and dental cement. Care is taken not to interfere with scalp circulation during retraction or to damage musculature of the neck. Injection of Lidocaine is administered subcutaneously along the wound site to reduce post-surgical pain. Ketoprofen (SC 5-10 mg/kg) is administered prior to recovery from anesthesia for post-surgical pain management. During recovery the animal is placed on a heating pad and is constantly monitored until effects of anesthesia have worn off. Following recovery from surgical implantation, animals are placed in a cage with clean bedding. Additional Ketoprofen will be administered twice daily over 48 hours post-surgery and animals are monitored for a minimum of 96 hours for post-surgical complications.

Euthanasia

Following completion of experimental manipulations, animals will be euthanized with an overdose of sodium pentobarbital (I.P., 120 mg/kg) which is consistent with the recommendations of the 2013 AVMA Guidelines on Euthanasia.

Multi-PI Leadership Plan

We are proposing a team science approach to develop a realistic multiscale circuit model of the larval zebrafish brain and explore how it generalizes to other animal models. Meeting this enormous challenge is not only well beyond what can be achieved in a single laboratory, it requires a collaboration of equals. Our team of three project directors (PDs)-Florian Engert, Jeff Lichtman, and Haim Sompolinsky--will share the responsibility and authority for leading and directing the project. We are submitting this proposal under the multiple-PI model.

Conceptually, the expertise of these PIs together covers neural circuit structure (Lichtman), circuit function and behavior (Engert), and modeling (Sompolinsky). Each of these three legs is essential, and the overall project needs these three to form a coherent leadership group. The multiple-PI model will ensure equal overall responsibility of all 3 PIs, deepen their interactions, and enhance the synergy of their work. These PIs have already established excellent working relationships, as reflected in their previous collaborations, including the U01 research project that helped launch the project proposed here.

This troika will work closely together to lead the Project. Engert and Lichtman have their labs in neighboring buildings at Harvard. Sompolinsky's office is in the same suite as Lichtman's. Sompolinsky is resident at Harvard for half of each year. For the other half of the year, while he is in Jerusalem, he is in close contact by Skype and email. Sompolinsky has been a long-term visiting professor at Harvard for the past decade, with an active group of postdocs and graduate students here, and has worked out many ways to remain in close contact with his group and his Harvard colleagues.

The leadership group will meet every two weeks to ensure that the Project is proceeding smoothly. In addition, as part of the Administrative Core, we will assemble an Internal Advisory Committee (IAC) to oversee the smooth coordination of efforts among all the components. Each member of the IAC has primary responsibility for one Project or Resource Core.

- Florian Engert will direct the Administrative Core, the Atlas Project, and the Behavioral and Imaging Project.
- Jeff Lichtman will direct the EM Core and Live Imaging Core.
- Haim Sompolinsky will direct the Theory and Modeling Project.
- Joshua Vogelstein will direct the Data Core.
- Aravi Samuel will direct the Engineering Core.
- Bence Ölveczky will direct the Other Model Systems-General Principles Project.

The IAC will meet monthly to discuss project progress and future project and administrative plans. The IAC will discuss any changes in the direction of the research projects and the re-budgeting of funds, if necessary. Entela Nako will coordinate this meeting and will also advise the IAC about the progress each group is making towards predetermined milestones and project goals against the expected timeline.

When conflicts arise in the full Team, the IAC will resolve them. Any Team investigators who are not on the Committee and who feel they are being treated unfairly will be invited to email their complaint to the Administrative Director for distribution to the Internal Advisory Committee listed above. A prominent notice to this effect will be posted on the Project web site.

When there is division within the IAC, after discussion the matter will be put to a vote in which a simple majority will prevail. In the case of a voting tie, the troika of PIs will make the final decision. If conflicts or disagreement persists after a decision is made, the committee will discuss the issue with the appointed ombudsman, David Cox, who will advise on the best course of action for the resolution of the conflict (see letter of support for letter from Cox).

The Internal Advisory Committee will work closely with the grant administrators at Harvard as well as the administrators at the other institutions to monitor all administrative aspects of the U19 grant. Our grant administrators, Doreen Charbonneau and Entela Nako, have previous experience in managing the U01 grant and have been working together to prepare submission of the U19 grant. Additionally, they have been working closely with the grant managers from our collaborating institutions to facilitate cooperation on the preparation of the proposal submission packet. They will continue to work closely with these colleagues at other institutions for the duration of the grant to ensure adherence to NIH policies, monitor finances, keep spending within the predetermined budgets, and help with the preparation of progress reports. To this end, the grant administrators will have a monthly conference call to discuss progress to date and any other issues that might arise.

Finally, we welcome the establishment of a to-be-named External Advisory Board (EAB) to be convened annually to assess progress and accomplishments, and to advise the team governance and participating NIH science officers. We will work closely with the EAB to ensure successful completion of our project. Entela Nako will coordinate this annual meeting.