SEEING THE WHOLE PICTURE: A COMPREHENSIVE IMAGING APPROACH TO FUNCTIONAL MAPPING OF CIRCUITS IN BEHAVING ZEBRAFISH

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Abstract—In recent years, the zebrafish has emerged as an appealing model system to tackle questions relating to the neural circuit basis of behavior. This can be attributed not just to the growing use of genetically tractable model organisms, but also in large part to the rapid advances in optical techniques for neuroscience, which are ideally suited for application to the small, transparent brain of the larval fish. Many characteristic features of vertebrate brains, from gross anatomy down to particular circuit motifs and cell-types, as well as conserved behaviors, can be found in zebrafish even just a few days post fertilization, and, at this early stage, the physical size of the brain makes it possible to analyze neural activity in a comprehensive fashion. In a recent study, we used a systematic and unbiased imaging method to record the pattern of activity dynamics throughout the whole brain of larval zebrafish during a simple visual behavior, the optokinetic response (OKR). This approach revealed the broadly distributed network of neurons that were active during the behavior and provided insights into the fine-scale functional architecture in the brain, inter-individual variability, and the spatial distribution of behaviorally relevant signals. Combined with mapping anatomical and functional connectivity, targeted electrophysiological recordings, and genetic labeling of specific populations, this comprehensive approach in zebrafish provides an unparalleled opportunity to study complete circuits in a behaving vertebrate animal.

Key words: zebrafish, whole-brain imaging, neural circuits, behavior, sensorimotor circuits.

THE CHALLENGE OF BRIDGING SCALES IN SYSTEMS NEUROSCIENCE

Our brains must continuously integrate information from the senses, past experience and internal states to plan and execute appropriate behaviors. A central aim of systems neuroscience is to understand how activity dynamics in the complex, distributed neuronal networks in the brain contribute to carrying out these tasks. This is a particularly challenging problem because it requires an integrated understanding of processes that span scales which may differ by orders of magnitude (van Hemmen and Sejnowski, 2005; Grillner, 2014), from the biophysical properties of individual cells to networks of billions of interconnected neurons. Frequently, however, technical limitations constrain analysis to one particular level. For example, electrophysiology allows recordings with very high fidelity and temporal resolution, but these recordings are usually limited to one or a few neurons in a restricted area. On the other hand, imaging methods that measure activity patterns throughout the whole brain, such as functional magnetic resonance imaging (fMRI), can typically report only the pooled activity of many neurons. Recent studies that applied in vivo calcium imaging to the transparent brains of Caenorhabditis elegans and zebrafish have shown great potential to bridge this

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gap by imaging large fractions of the brain at single-cell resolution (Ahrens et al., 2013; Panier et al., 2013; Schrödel et al., 2013; Portugues et al., 2014; Prevedel et al., 2014).

IDENTIFYING THE CIRCUITS UNDERLYING BEHAVIORS

The ability to obtain a whole-brain perspective is valuable since even an extremely simple behavior may involve several groups of neurons scattered throughout many different brain areas. How can we identify the distributed networks responsible for a particular behavior? One approach is to trace out pathways anatomically, as exemplified by the burgeoning field of connectomics, which aims to comprehensively map connectivity among large assemblies of neurons or even throughout whole brains. The connectome of *C. elegans* has been available for some time (White et al., 1986), and this information has acted as a powerful guide, providing constraints which have enabled rapid progress in the identification of circuits underlying important functions such as sensory processing, locomotor control and learning (Chalfie et al., 1985; Bargmann and Horvitz, 1991; Mori and Ohshima, 1995; Tsalik and Hobert, 2003; Gray et al., 2005; Ha et al., 2010). More recently, it has become possible to apply this approach on a sufficiently large scale to map the connections in substantial volumes of brain tissue in both the rodent and fly visual systems (Helmaatseder et al., 2013; Takemura et al., 2013). At the same time, the description of the structure of networks is not, by itself, sufficient to reliably predict circuit function, since even a very complete map will lack essential pieces of information needed to predict the resulting activity dynamics, including intrinsic electrical properties of neurons, the relative strengths of different connections, and the impact of modulatory inputs (Bargmann and Marder, 2013).

The more common approach to identifying the cells involved in a specific task has been to record neural activity during behavior or passive presentation of sensory stimuli, and relate this activity to the ongoing computations and task demands. This has typically been done using recordings with extracellular electrodes (Adrian, 1926), and usually from one neuron at a time. In this way, it is possible to identify neurons that are tuned to properties of sensory stimuli (Hubel and Wiesel, 1959), or motor output (Georgopoulos et al., 1982), or whose firing reflects internal parameters linked to the ongoing computations in different regions of the brain (Goldberg and Wurtz, 1972). Although single-unit recordings account for much of our knowledge regarding the signals carried in different brain areas, more information can often be extracted when data from several neurons are analyzed together (Perkel et al., 1967; Brown et al., 2004; Churchland et al., 2007; Miller and Wilson, 2008). Broad and overlapping tuning curves will mean that representations are distributed over many cells (Erickson, 1968; Georgopoulos et al., 1986; Young and Yamane, 1992), and the temporal evolution of the population dynamics may not be evident in the responses of single neurons (Friedrich and Laurent, 2001; Briggman et al., 2005; Mante et al., 2013; Kaufman et al., 2014). In many such cases, the distribution of activity in the population may nevertheless be established over many sequential single-unit recordings. However, if important information is encoded in the covariance between neurons, or in patterns of activity that are not faithfully repeated from trial to trial, it is necessary to record from two or more cells simultaneously (Zohary et al., 1994; Meister et al., 1995; Nicolelis et al., 1995; Singer and Gray, 1995; Vaadia et al., 1995; Harris et al., 2003; Jones et al., 2007). While electrophysiological methods have been developed to record from many neurons (Nicolelis et al., 1993; Meister et al., 1994), *in vivo* calcium imaging is becoming an increasingly popular tool for population recordings, particularly thanks to the development of genetically encoded indicators (Miyawaki et al., 1997; Nakai et al., 2001), which have undergone rapid recent improvements in sensitivity and speed (Chen et al., 2013; Sun et al., 2013; Thesstrup et al., 2014). While the fidelity and temporal resolution of calcium imaging may not yet be a match for the electrode, there are several advantages which make it a very useful approach, including:

1. Large populations of neurons can be imaged at once, which allows high experimental throughput, and also reduces recording bias. Importantly, it may also reveal aspects of information processing that are not captured by serial recordings from one or a few cells, as discussed above.

2. Recordings can be restricted to genetically defined populations, including different neurotransmitter classes, by cell-type specific expression of the indicator, or a fluorescent co-label (Diez-Garcia et al., 2005; Sohya et al., 2007; Yaksi et al., 2007; Kerlin et al., 2010); and it is even possible to selectively record the pooled activity from one class of neurons (Naumann et al., 2010; Cui et al., 2013). Reading signals from genetically specified neurons has also become possible, to some degree, by using electrophysiology in combination with optogenetic stimulation (Lim et al., 2009).

3. Precise spatial information is retained, both in the arrangement of neuronal cell bodies, and fine structural organization in neuropil (Ohki et al., 2005; Komiyama et al., 2010; Nikolaou et al., 2012).

4. Subcellular signaling can be resolved, allowing the measurement of both spatial aspects of dendritic processing (Borst and Egelhaaf, 1992; Svoboda et al., 1997; Euler et al., 2002; Hill et al., 2013) and the topography of synaptic inputs (Baden and Hedwig, 2007; Bollmann and Engert, 2009; Peron et al., 2009; Hopp et al., 2014).

5. Recordings can be made in a minimally invasive manner, especially in transparent organisms, in some cases while they are freely moving (Clark et al., 2007; Ben Arous et al., 2010; Naumann et al., 2010; Faumont et al., 2011; Piggott et al., 2011; Larsch et al., 2013; Muto et al., 2013).

6. Information about cell morphology can be obtained simultaneously, and the recorded cell can be
THE ZEBRAFISH MODEL SYSTEM

Given the benefits of using optical methods to record and also manipulate neural activity, zebrafish have emerged in recent years as a promising model organism in systems and circuit neuroscience (Friedrich et al., 2010). Their neural development is rapid, with the first retinal ganglion cell (RGC) axons leaving the eye at 34-h post-fertilization (Stuermer, 1988) and behavioral responses to visual stimuli appearing within the first 3 days of life (Easter and Nicola, 1996). One-week-old larvae, just four millimeters in length, can follow stimuli with their eyes, track and capture small, moving prey, avoid predators and stabilize their position in moving water (Easter and Nicola, 1997; Neuhauss, 2003; Portugues and Engert, 2009). Many of these behaviors can be reproduced in head-restrained larvae, and, by using appropriately timed, closed-loop presentation of visual feedback, it is even possible to elicit naturalistic sequences of coordinated movements (Portugues and Engert, 2011; Trivedi and Bollmann, 2013). At this early stage, the zebrafish brain consists of roughly 100,000 neurons, and is transparent and sufficiently small, measuring about 800 x 400 x 300 μm, that the whole volume can be imaged at subcellular resolution within the field of view of a typical microscope. Moreover, recent advances in transgenic technology mean that new stable lines with cell-specific expression of genetic tools can be generated cheaply and rapidly (Abe et al., 2011; Suster et al., 2011).

SELECTED ZEBRAFISH CONTRIBUTIONS TO SYSTEMS AND CIRCUITS NEUROSCIENCE

Thanks to these advantages, the zebrafish model has been used to address important questions in many different areas of systems and circuits neuroscience. While this is not intended to represent a comprehensive list (functional imaging studies in zebrafish are described more completely elsewhere: Kettunen, 2012; Renninger and Orger, 2013), we highlight below a few examples of important recent discoveries and observations that originated in the zebrafish model, which have potentially broad relevance for neural circuit function in other organisms.

Retinal processing

Imaging has been used to reveal novel aspects of synaptic function in the zebrafish retina, with implications for visual processing. Using a synaptically targeted indicator, Dreosti et al. made the surprising discovery of all-or-nothing calcium spikes at the bipolar cell synapse, which were precisely time-locked to visual stimuli, challenging the textbook view of graded transmission (Baden et al., 2011; Dreosti et al., 2011). Further investigations revealed a surprising triphasic relationship between luminance changes and vesicle release in some bipolar cell terminals, and suggested a role for this unusual response in efficient coding of fluctuating light stimuli (Odermatt et al., 2012). In another study (Wang et al., 2014), some long-standing questions surrounding the mechanistic origin of lateral inhibitory signals from horizontal cells to photoreceptor terminals were addressed. Using a genetically encoded pH sensor targeted to the synaptic cleft of cone terminals in zebrafish, it was shown that light-evoked synaptic alkalinization due to a change in proton flux across horizontal cell membranes is sufficient to mediate this process (Wang et al., 2014).

Circuit mechanisms of vision

RGCs in zebrafish project to at least 10 arborization fields (AFs) with the vast majority innervating the optic tectum (AF 10) (Burnill and Easter, 1994). Within the tectum, RGCs terminate in four different layers. Multicolor labeling of single axons using Brainbow (Livet et al., 2007) demonstrated that RGC arbors, can be further separated into at least 10 distinct sublaminae, each a few microns in thickness (Robles et al., 2013). Each sublamina receives input from a distinct combination of RGC types, suggesting that the tectum may receive segregated input from parallel visual processing streams. Functional studies revealed that RGCs sensitive to different directions of whole-field motion target different tectal sublaminae (Gabriel et al., 2012; Nikolau et al., 2012), and that neurons in the tectum integrate these inputs to generate distinct tuning characteristics (Hunter et al., 2013).

The tectum is not required for the optokinetic response (OKR) (Roers and Baier, 2003), but Kubo et al., using optogenetic activation and silencing, identified a region, in the vicinity of retinal AF 9, that is both necessary and sufficient to drive smooth eye movements (Kubo et al., 2014). Imaging systematically from cell bodies surrounding this area, in the area pretectalis, in response to different combinations of horizontal motion presented to the two eyes, they identified several classes with different response profiles. These included both monocular neurons, and binocular neurons sensitive to same-direction (“translational”) and opposite direction (“rotational”) motion between the two eyes. Importantly, because they had a comprehensive picture of the functional types in the population, and their locations, they were able to propose, based on minimal Boolean logic, the simplest connectivity pattern that could explain the observed responses, providing a straightforward circuit hypothesis which can now be tested experimentally (Kubo et al., 2014).

Spinal cord motor circuits

As reviewed extensively elsewhere (Fetcho and McLean, 2010), a large body of work has established fundamental principles of recruitment of spinal neurons during different modes of locomotion (McLean et al., 2007, 2008). Recent evidence has indicated that motor neuron pools in zebrafish are divided into discrete modules with different patterns of recruitment and muscle innervation, a finding with important implications for motor control as well as the evolution of more complex locomotor circuits.
The small size of zebrafish has allowed three recent studies to investigate the supraspinal control of locomotion by assessing the contribution of a small midbrain nucleus of spinal projecting neurons to both postural control and swimming speed (Severi et al., 2014; Thiele et al., 2014; Wang and McLean, 2014). These studies show how the interplay between the axonal projection patterns of these specific neurons and variations of biophysical properties of spinal neurons along the dorsal–ventral axis result in the implementation of specific locomotor maneuvers and how identified neurons can contribute selectively to modulation of different parameters of behavior.

### Brainstem motor circuits

While the relationship between domains of transcription factor expression and neuronal identity has been well established in the spinal cord (Briscoe et al., 2000; Goulding et al., 2002), it was unclear if and how the same principles could be applied to circuits in the hindbrain. Kinkhabwala and colleagues demonstrated that transcription factor stripes extend from the spinal cord into the medulla, but display a medio-lateral rather than dorsal-ventral organization (Kinkhabwala et al., 2011). Each stripe is associated with neurons of a particular morphology and neurotransmitter identity, such as glutamatergic ipsilateral descending neurons. Using kaede photoconversion to label the birth order of neurons, they found that each stripe showed a dorso-ventral gradient of age. Furthermore, targeted electrophysiological recordings showed that there was a striking relationship between the tail-beat frequency at which neurons were recruited during fictive swimming and their location, with older, more ventral, neurons becoming active only during higher frequency bouts of swimming. From this observation, they propose a model in which circuits for different behaviors develop in a temporal sequence, each drawing from a pool of available neuron types originating from the different expression zones (Kinkhabwala et al., 2011). In a companion study, Koyama et al. combined systematic paired patch recordings, and confocal reconstructions of neuronal morphology, to reveal how a circuit mediating Mauthner-cell initiated escapes is constructed from this modular architecture (Koyama et al., 2011). Interesting insights from the zebrafish into the mechanisms of neural integration in the brainstem circuits mediating eye movements are reviewed in detail elsewhere in this issue (Joshua and Lisberger, 2014).

### Comprehensive imaging from neural populations

Several studies have taken advantage of the small size of the zebrafish brain to make comprehensive recordings of activity from particular populations of neurons. In an adult brain preparation, Yaksi and colleagues mapped the spatiotemporal dynamics of responses throughout most of the olfactory bulb (Yaksi et al., 2007). Analysis of the temporally deconvolved population responses showed that an initially chemotopic output pattern evolved rapidly into a sparse representation of odor identity. Repeating a similar approach for olfactory target structures in the telencephalon (Yaksi et al., 2009), they showed differences in coding between subpallial and pallial regions, with the former showing broad odor tuning, and the latter containing cells that responded more specifically to particular odor combinations.

The habenula, a key relay station between the forebrain and neuromodulator systems, has received considerable attention in the zebrafish, due to its pronounced asymmetries in morphology, gene expression, innervation, axonal projections and functional responses (Concha et al., 2000; Hendricks and Jesuthasan, 2007; Kuan et al., 2007; Bianco et al., 2008; Miyasaka et al., 2009; deCarvalho et al., 2014; Dreosti et al., 2014), as well as its apparent central role in determining behavioral choices (Agetsuma et al., 2010; Lee et al., 2010). Krishnan et al. developed a simple, wide-field epifluorescence system for rapid three-dimensional imaging using fast focusing and deconvolution, and applied this method to reveal, with single-cell resolution, the dynamics in response to different concentrations of multiple odors throughout the whole habenula (Krishnan et al., 2014). They found that population activity in the right dorsal habenula varied with the concentration of a socially relevant odor (a bile salt), and provided evidence, using pharmacology and ablations, that this region mediates a switch from attraction to avoidance at high concentrations.

In addition to revealing spatial patterns of activity, volume imaging from individual animals provides an unbiased method to identify rare or sparsely distributed cell types. Previous work by Orger and colleagues aimed to determine which reticulospinal cells in the zebrafish brainstem were active when the fish was swimming forward or turning (Orger et al., 2008). They imaged systematically through the whole population while presenting stimuli moving in different directions. While many cells were activated by forward motion, only a few were preferentially activated by leftward and rightward motion, and these were small and weakly labeled, and could easily have been overlooked by a more selective sampling strategy. Knowing the spatial locations of all the cells that were active during turns, it was possible to systematically ablate them, and show that the fish could no longer perform optomotor turns toward the ablated side (Orger et al., 2008). Subsequent studies showed that the same ventral neuron groups are required, in general, for the fish to make routine turns, for example during spontaneous swimming or phototaxis (Huang et al., 2013). Since the set of neurons associated with a particular type of swim may be distributed across several reticulospinal groups, the chances of ablating exactly the right combination to specifically eliminate a single behavior, in the absence of a functional map, may be very small. Moreover, knowing the context in which the cells are active makes it possible to assay a more targeted set of behaviors and identify more subtle phenotypes (Liu and Fetcho, 1999; Seveni et al., 2014).

Some of the greatest potential the zebrafish model offers lies in the ability to monitor population activity across multiple brain regions, or even throughout the...
entire brain. In one study, different brain volumes were imaged across many paralyzed fish, during adaptation of the fictive optomotor response (OMR) to different closed-loop gains (Ahrens et al., 2012). Activity patterns were correlated with the visual motion, gain changes or fictive motor output and the resulting data were subsequently aligned to a reference brain with an accuracy of 20–25 μm to yield brain-wide correlation maps. As described in more detail below, we recently imaged activity through most of the fish brain at micron resolution, generating whole-brain functional maps from individual animals performing optokinetic behavior, while partially restrained (Portugues et al., 2014). Light-sheet imaging, which allows for faster acquisition rates than two-photon laser scanning microscopy, can be used to acquire data nearly simultaneously from large portions of the brain (Panier et al., 2013). This approach allowed the recording of spontaneous whole-brain activity from agarose-embedded larvae at around 1 Hz, resulting in the identification of functionally defined three-dimensional structures spanning multiple regions (Ahrens et al., 2013), and is compatible with simultaneous recording of fictive visual behavior (Vladimirov et al., 2014). Light-field imaging is an approach that promises even faster volume acquisition rates, by capturing the data from multiple focal planes in a single camera exposure (Levoy et al., 2009; Broxton et al., 2013). This method was successfully applied recently to functional imaging in both worms and zebrafish (Prevedel et al., 2014), although several critical hurdles, such as sample bleaching and very long data processing times, still remain to be addressed.

WHOLE-BRAIN IMAGING OF ZEBRAFISH LARVAE DURING OPTOKINETIC BEHAVIOR

The OKR is a reflexive behavior that consists of a smooth, tracking eye movement in response to whole-field rotational motion, interrupted by fast reset saccades, and is thought to serve to reduce or cancel retinal slip. This behavior is found throughout the animal kingdom (Walls, 1962; Masseck and Hoffmann, 2009), and, although every type of animal has different constraints and specializations, for example due to foveal vision or lateral vs. frontal eyes, the sensorimotor transformation that occurs during OKR presumably relies on similar circuit and neural computations across species. In a recent study (Portugues et al., 2014), we set out to map neural activity dynamics with single-cell resolution through the whole brains of fish while they performed this behavior, reasoning that such a comprehensive map would be a significant step toward understanding the organization and function of the underlying network.

The OKR appears in zebrafish at an early stage, and is reliably evoked by a rotating pattern of vertical stripes at 5 days post-fertilization (Easter and Nicola, 1997; Beck et al., 2004). We imaged from awake, partially restrained larvae with pan-neuronal expression of the genetically encoded calcium indicator GCaMP5G (Akerboom et al., 2012), while they responded to sinusoidally rotating patterns which elicited the OKR (Fig. 1A, B). Our custom-built setup allowed the brain to be stably imaged using two-photon excitation, while the eyes and tail were free to move. The fish’s behavior was tracked with a camera, revealing robust and consistent responses over many hours (Fig. 1B). Therefore, we could gather data sequentially from hundreds of planes, under similar behavioral conditions, sampling the whole brain at less than 1 μm resolution in all three dimensions. Most cells in the brain, at this age, have cell bodies 3–6 μm in diameter. Fig. 1C shows color-coded maps of response magnitude, and phase relative to the stimulus, at various depths in the brain, superimposed on a grayscale image of the brain anatomy. Individual cell bodies can be clearly distinguished, based on nuclear exclusion of the GCaMP, and groups of active voxels that colocalize with single neuronal somata, as well as with neuropil structures, are evident. In addition to the fluorescence time-series for each voxel that describes the neuronal activity, each sequentially recorded slice is accompanied by the time-course of the stimulus presented and high-speed recordings of behavior. From 400 to 600 such planes, we can build three-dimensional maps of the average activity dynamics, composed of around 200 million voxels. Fig. 1D shows a projection from two viewpoints of all identified active regions in an example fish, color-coded by response phase.

Although phase-locked responses were detected in only a small percentage of the neurons in the imaging volume (<5% of voxels imaged), the active areas were widely dispersed. Responses were found in structures throughout the brainstem including multiple retinal ganglion AFs, the periventricular layers of the optic tectum, and areas in the hindbrain such as the cerebellum and the inferior olive, even spanning regions in the forebrain such as the habenula (Fig. 1C, D). At the same time, even within individual regions, the activity pattern could be quite sparse. For example, although activity was reliably observed in the optic tectum, responses were restricted to much less than 1% of all neurons. The sparse and broadly distributed nature of the network that is engaged during a relatively simple, reflexive behavior, such as the OKR, highlights the benefits of being able to record activity systematically throughout the brain.

Neuronal responses were locked to different phases of the stimulus, with different brain areas showing distinct phases of activation relative to the rotating stimulus (Fig. 1B–D). Areas that receive input from the retina and are therefore likely engaged in the representation of sensory information, such as the tectum and pretectum, responded earlier in the stimulus cycle than areas associated with motor output, revealing the dynamics of information flow across brain regions during this behavior.

Imaging methods, as compared to electrode recordings, not only provide information about the functional properties of different areas, but they also allow precise spatial mapping of the responses. Voxelwise analysis of the response phase shows that activity within most regions is not synchronous, but instead shows smooth spatial gradients of activation. Taken together
with observations of gradients of physiological and functional properties in the zebrafish spinal cord, brainstem and oculomotor circuitry (Fetcho and McLean, 2010; Kinkhabwala et al., 2011; Miri et al., 2011a), this suggests that such functional topography may be a general organizing feature of sensorimotor circuits.
COMPARING ACTIVITY ACROSS INDIVIDUALS—STEREOTYPY OF NEURONAL RESPONSES

The active areas, while widely dispersed, nevertheless showed a conspicuously ordered spatial arrangement, which is particularly evident when one compares the patterns on the left and right sides of the brain. The whole network has a striking symmetry, right down to individual neurons and groups of neurons, with opposite structures in the brain active 180° out of phase. The notable exception to this is in the dorsal habenula, where responses are heavily biased toward the left side, consistent with other work investigating the distribution of visual and olfactory signals in this structure (Dreosti et al., 2014). This led us to ask: how consistent is this pattern from one fish to another? In invertebrate systems, it is common to find identifiable neurons across animals (Selverston, 2010; for an example see O’Shea and Williams, 1974), and tracing of single axons in Drosophila, aligned to a reference brain, revealed that even long-range projections may show stereotyped organization, on the order of a few microns, across individual animals (Jeffries et al., 2007). In other cases, though, studies have found more random organization (Lu et al., 2009; Caron et al., 2013). To address this question, all the imaged brains were first aligned to a reference stack using a non-rigid deformation. We then asked, for each detected region of interest: how far do you have to travel, on average, in the brain of another fish to find a region active at a similar phase? For a large portion of the regions active during OKR, including the pretectum, cerebellum, habenula, and an extensive hindbrain network, this was around 1–5 μm, which is on the order of a single neuronal cell body, and indicates a high degree of stereotypy (Fig. 1E). This highly consistent organization across fish brains suggests that what we learn from an individual brain can, at least for simple behaviors such as the OKR, be straightforwardly extrapolated to other fish. One practical advantage of this is the ability to use the functional maps obtained to guide targeted ablation, imaging or photoactivation to areas of interest, or to compare with the distribution of molecular or genetic markers (Ronneberger et al., 2012). Moreover, the high degree of stereotypy allowed us to combine data from multiple fish, improving the signal-to-noise in areas that were weakly active, or dimly labeled, in individual fish, thus providing a more comprehensive map of activity during behavior (see Fig. 4 in Portugues et al., 2014).

LOCALIZING SENSORIMOTOR SIGNALS TO DIFFERENT BRAIN AREAS

Next, we asked how this pattern of activity reflects the processing of behaviorally relevant information in different parts of the network. When using this simple stimulus to drive the OKR, the sensory input and motor outputs are highly correlated, and the left and right eyes move together in a conjugate fashion. Therefore, in order to reveal what signals are present in different areas, we employed a richer stimulus set, in which the same basic sensory cues were presented, but in different combinations, giving rise to more variable and complex sequences of motor output (Fig. 2A). Taking into account the response kernel of GCaMP5G (Fig. 2B; see (Miri et al., 2011b)), we then constructed a set of variables, based on the properties of the sensory stimulus, measured motor outputs, and other behaviorally relevant parameters (we will refer to them as regressors, following Miri et al., 2011b, although here we are measuring the correlation of each of these variables with imaging data). Local regions of activity could be identified which correlated strongly with different regressors related to both sensory and motor features including eye position, stimulus velocity and swimming episodes (Fig. 2C), as well as intermediate steps of sensorimotor processing. For example, we made the unexpected observation that some wide-field motion-selective neurons in the optic tectum appear to integrate information from the two eyes, although the tectum only receives direct inputs from the contralateral eye. These neurons responded phasically when the direction of motion to the two eyes was the same, consistent with translational movement, but their responses were suppressed when motion occurred in opposite directions during a rotating stimulus, similar to some neurons in the area pretectalis described above (Kubo et al., 2014).

Using our data sets we could then examine, in an unbiased manner, how these signals are distributed through the brain, and compare this distribution across animals. We extracted the fluorescence time courses for an array of overlapping ~5 μm cubes tiling the whole imaging volume, and identified the best matching regressor for each. Voxels matching particular regressors were tightly localized to particular areas, with very few found outside a few dense regions. These locations were also highly consistent between fish. Fig. 2D shows superimposed projections, from dorsal and anterior views, of all voxel locations correlated with example sensory and motor variables, which were identified in the brains of seven individual fish. The distributions form either matched lateralized pairs of clusters, as shown for left eye and right eye position signals, as well as left and right side stimulus velocity, or broad symmetric structures, as shown for swimming-related activity. Thus, the broadly distributed pattern of activity shown in Fig. 1D can be decomposed into local modules subserving particular aspects of the sensorimotor task.

CONCLUSIONS

The ability to rapidly identify which neurons are active during a particular behavior, or constitute specific functional classes, even when they are very few, or are distributed across a wide area, provides a powerful head start in deciphering the circuit mechanisms that underlie the behavior. However, it is important to recognize that such mapping studies are not, by themselves, a solution to such questions, but instead are a foundation for further investigations. Essential next steps will be to determine the molecular genetic identity, morphology and connectivity of the identified neurons,
and to demonstrate through gain- and loss-of-function experiments what role they actually play in shaping the observed responses.

Many transgenic lines exist which allow expression of genes in particular classes of neurons. These may be generated by random enhancer trapping (Scott et al., 2011b; Portugues et al., 2014).
2007; Asakawa et al., 2008), or by directed attempts to label populations expressing particular genetic markers (Suster et al., 2009). For hindbrain circuitry, systematic sets of driver lines have been generated which target neurons based on both the transcription factor domains that define different basic neuronal classes and also many of the important neurotransmitter systems (Kinkhabwala et al., 2011; Satou et al., 2013). Performing the same mapping studies in fish where these populations are specifically labeled will not only provide more detailed information on the morphology and projections of the different neurons, but also useful information on their possible circuit function: are they excitatory or inhibitory? Do they have commissural axons? The fact that the activity patterns in many regions are highly stereotyped, down to the level of a few microns, is helpful from the perspective of identifying useful driver lines. Collections of high-resolution, 3D-maps of gene expression patterns, aligned to a standard brain (Ronneberger et al., 2012), can be compared with similarly aligned functional maps, given a “bridging” transformation between their respective reference frames, making it possible to quickly search for lines whose expression falls in areas of interest.

Within the network of active neurons correlated with different aspects of a particular behavior, it will be critical to be able to up- and down-regulate activity in specific subpopulations in order to identify which of them play a direct role in shaping the behavioral response, and to test hypotheses about circuit organization. Genetic ablations can be a powerful way to target a defined population, providing a very specific promoter exists, and can even be executed at defined developmental stages using nitroreductase, an enzyme which produces cytotoxic products when provided with an artificial brain areas has been shown in fish as old as 4 weeks (Jetti et al., 2014), at which age fish do begin to show a mature capacity for learning (Valente et al., 2012). It is possible, using more invasive preparations, to image neuronal populations in adult zebrafish and this approach was used to record changes in population activity in telencephalic areas proposed to be part of a circuit homologous to the mammalian cortico-basal ganglia loop (Aoki et al., 2013). In that study, the authors identified areas in the dorsal telencephalon where cue-related activity appeared following training. Interestingly, the precise area which became active for a particular cue was dependent on the task contingencies the fish associated to that cue.

An interesting line of investigation will be to compare the sets of neurons activated during different behaviors in the same animal. Are there pathways dedicated to particular responses, or do different behaviors emerge from the patterns of responses in common sets of neurons (Shaw and Krishnan, 2014)? At the motor end, evidence suggests that different swimming behaviors in zebrafish may share common reticulospinal output pathways (Sankrithi and O’Malley, 2010; Huang et al., 2013), and, similarly, behaviors that result in eye movements are likely to converge on a common oculomotor system (Büttner-Ennever and Horn, 1997). At the sensory end, distinct systems may process particular general types of visual information, which are important for many different behaviors. The optomotor and OKRs are, respectively,
swimming and eye-tracking behaviors, which are elicited by similar global motion patterns. The area pretectalis neurons imaged by Kubo et al. show a diverse array of responses to combinations of horizontal whole-field motion presented to the two eyes, and it is proposed that they provide input for both the OKR and OMR (Kubo et al., 2014). How behavioral decisions emerge from the dynamic interactions of many interconnected local networks of neurons, each dedicated to particular functions, is a critical, but relatively unexplored, question in neuroscience (Sompolinsky, 2014). Addressing it will likely require that we measure activity in large populations of neurons, both within and across areas. It has been demonstrated for behaviors ranging from invertebrate sensory-motor choices to complex cognitive tasks in primates that, to understand either the mechanisms of decision making within local circuits, or the functional coupling of information across areas, it is often necessary to look at whole population activity, rather than single neurons (Briggman et al., 2005; Mante et al., 2013; Kaufman et al., 2014).

The ability, in zebrafish, to record simultaneously both local and global population activity makes it a promising system to address this question.

In summary, we have described a comprehensive imaging approach in behaving zebrafish, that can be applied to many different behaviors. This has allowed us to explore the functional architecture of the zebrafish brain, delineating areas involved in sensory processing, motor generation, or the combination and transformation of information that happens in between. A comprehensive knowledge of the distribution of functional classes in these regions makes it possible to trace the flow of information from one area to the other, and generate hypotheses about the possible circuit connectivity, which can then be tested with more targeted electrophysiological or anatomical tracing experiments. In the future, the ability to study behavioral circuits that are defined from sensory input to motor output, to monitor single-neuron activity throughout the whole brain and to optically manipulate individual cellular components will make the zebrafish a very powerful system to study the circuit mechanisms underlying behavior.

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