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Current Opinion in Neurobiology

Integrative whole-brain neuroscience in larval zebrafish

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Due to their small size and transparency, zebrafish larvae are amenable to a range of fluorescence microscopy techniques. With the development of sensitive genetically encoded calcium indicators, this has extended to the whole-brain imaging of neural activity with cellular resolution. This technique has been used to study brain-wide population dynamics accompanying sensory processing and sensorimotor transformations, and has spurred the development of innovative closed-loop behavioral paradigms in which stimulus–response relationships can be studied. More recently, microscopes have been developed that allow whole-brain calcium imaging in freely swimming and behaving larvae. In this review, we highlight the technologies underlying whole-brain functional imaging in zebrafish, provide examples of the sensory and motor processes that have been studied with this technique, and discuss the need to merge data from whole-brain functional imaging studies with neurochemical and anatomical information to develop holistic models of functional neural circuits.

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Current Opinion in Neurobiology 2018, **50**:136–145

This review comes from a themed issue on **Neurotechnologies**

Edited by **Polina Anikeeva** and **Liqun Luo**

https://doi.org/10.1016/j.conb.2018.02.004

Population-scale imaging of neural activity in zebrafish larvae

The human brain comprises hundreds of distinct structures, thousands of cell types, billions of neurons, and trillions of connections, and understanding its function is one of the most daunting challenges facing the scientific community. Observations of brain function initially relied on lesions, either occurring sporadically in humans or in a targeted fashion in animal models, that allowed inferences about the brain regions or tracts necessary for producing behavior. More recently, large scale imaging techniques such as fMRI have revealed broad patterns of activity that coincide with perception, thought, or behavior. For more than half a century, electrophysiology has permitted the fine-grained analyses of the functions of individual neurons. Although these approaches have made enormous contributions to our understanding of the brain's functional architecture, a gap exists between large scale techniques, which have difficulty reporting on activity in individual neurons, and electrophysiology, which gives detailed information on the activity of a relatively small number of neurons. Neither reveals patterns of activity spread across the dozens, hundreds, or thousands of individual neurons whose orchestrated activity contribute to perception and behavior.

Recent advances in protein engineering and fluorescence microscopy have converged to make the observation of neural activity across large populations of neurons possible. The first step in this process was the development of genetically-encoded fluorescent indicators of physiological events (principally voltage or calcium flux) that reflect neural activity (reviewed by [1], and compared in Table 1). The fluorescent signals from these probes were initially detected with 2-photon microscopes, but more recently, selective planar illumination microscopy (SPIM) [2–5] and volumetric imaging techniques [6–9] have provided faster alternatives. Each of these indicators and imaging approaches comes with its advantages and limitations (outlined in Table 1), and different combinations are appropriate for different biological questions.

These genetically-encoded indicators and imaging techniques provide a framework for observing activity across populations of neurons with cellular resolution, but experiments still depend on the biological properties of the model organism. Zebrafish gained popularity as a model system in the 1990s, when they were used principally for developmental studies. In addition to generally desirable properties (small size, large broods, and more recently nimble genetics), their utility to developmental biologists sprang from a pair of inherent biological properties: they develop externally and are transparent at early life stages. At the time, few foresaw how beautifully these attributes would dovetail with the more recent optophysiological techniques outlined above [22]. Following a number of studies tracking activity across populations of neurons in specific parts of the larval zebrafish brain, this approach was eventually used to image activity across the entire brain with cellular resolution during behavior [23].

In this review, we will discuss subsequent studies involving whole-brain (or large-population) functional imaging

Table 1

An overview of the major strengths and limitations for popular methods of imaging neural activity

Method	Strengths	Limitations	Relevant references (select examples)
Imaging			
SPIM	High speed and large field of view. Relatively inexpensive. Basic open sourced configurations are simple to set up.	Illumination is orthogonal to the imaging, which some preparations will not tolerate. Visual stimulation of the larva from reflections from illumination plane, but see [10]. Stripe artifacts that mask responses or produce spurious signals. Requires a transparent/cleared sample and multiple objectives.	[2,5,11,12]
2-Photon	Lack of unintended visual stimulation of the larva. Deep tissue penetration with long wavelength light.	Slow speed, especially for volumes.	[13]
Extended depth of field light sheet microscopy	Fast volumetric imaging, no mechanical motion near sample	Same limitations as SPIM. Requires deconvolution of the images.	[8,14]
SCAPE	Fast volumetric imaging, no mechanical motion near sample, single objective	Comatic aberrations, slightly lower spatial resolution than native SPIM.	[6]
Light-field microscopy (LFM)	Fast volumetric imaging	Resolution and requires deconvolution	[7,15]
Indicators of activity			
Genetically Encoded Calcium Indicators (GECIs)	Good signal to noise ratio Slow kinetics aid in imaging volumes	Slow kinetics makes spike inference difficult. Difficult to infer temporal sequences of neural activity across populations.	[16,17]
Genetically Encoded Voltage Indicators (GEVIs)	Fast temporal kinetics	Low signal to noise ratio. High frame rates produce large files and complicate imaging large populations.	[18–21]

in larval zebrafish and the contributions that they have made toward characterizing sensory processing and sensorimotor behavior. We will also discuss this approach's limitations for testing the behavioral contributions made by the observed activity, and for gauging the functional circuits through which patterns of activity flow. Finally, we will discuss emerging technologies that, combined with population-scale functional imaging, may close the loop to provide holistic descriptions of functional circuits that span anatomy, connectivity, function, and behavior.

Observing neural pathways for sensory processing and the generation of behavior

One of the primary roles of the brain is to produce behavior, so measuring brain activity in behaving animals has obvious advantages. However, a traditional constraint of calcium imaging is imposed by its intolerance of motion. This restricts imaging to the brain's spontaneous activity [24–26] and sensory responses to modalities for which stimuli are compatible with a stationary animal (normally embedded in agarose, in the case of zebrafish larvae). These modalities include olfaction [27,28], audition [29–31], somatosensation [32], and most notably vision [33•,34–42]. Other modalities are fundamentally linked to the animal's movement through space, and these are more difficult to study in immobilized animals. The lateral line neuromasts, which are responsible for detecting water flow, can be stimulated with puffs of water in a tail-free preparation [29,30], but more realistic lateral line stimulation may be possible through microfluidics (Figure 1a–c). The vestibular system, tasked with detecting gravity and acceleration, poses particular challenges to functional imaging, although controlled tilting stimuli [43] may be compatible with calcium imaging on custom-built microscopes, and optical trapping of the otoliths has been shown to stimulate the vestibular system in stationary animals [44•] (Figure 1d–g).

Immobilized preparations also restrict behavioral outputs, although movements of the tail and eyes become evident if they are freed from the agarose that immobilizes the head. Distinct movements of the tail in such preparations can be interpreted as behavioral swimming, turning, postural correction, prey tracking, or startle, and this permits patterns of neural activity to be correlated to individual movements or combinations of movements representing more complex behaviors [34,45]. Similarly, movements of the eyes in immobilized larvae correspond to more complex behaviors in nature. In the case of the optokinetic response (OKR), the eyes sweep to follow