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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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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]

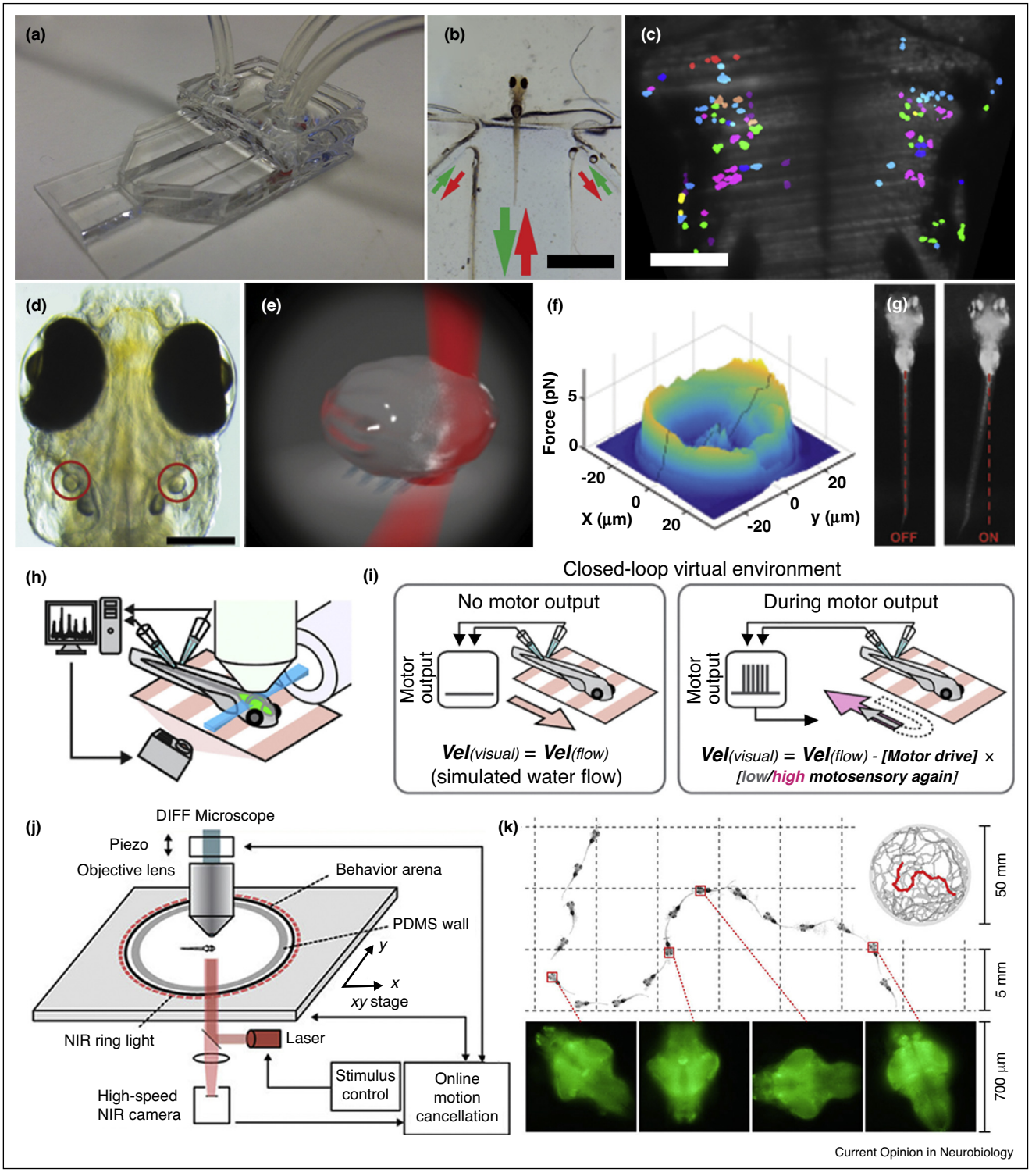
in larval zebrafish and the contributions that they have made toward characterizing sensory processing and sensorimotor behavior. We will also discuss this approach's limitations for testing the behavioral contributions made by the observed activity, and for gauging the functional circuits through which 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

Figure 1



Sensory stimulation during whole-brain imaging. **(a)** A microfluidics device in which a 6dpf larva can be mounted **(b)** to deliver forward (green arrows) or reverse (red arrows) water flow stimuli. Following calcium imaging and analysis, different categories of neural response (colors, **(c)**) can be mapped back onto the brain. The utricular otoliths (red circles **(d)**) mediate vestibular perception, so optical trapping (schematic in **(e)**, quantified in **(f)**) should mimic physical movement of the animal. Accordingly, larvae exposed to optical trapping (right **(g)**) show compensatory tail movements that are directional and scaled to the stimulus strength. **(h)** A closed-loop preparation with visual stimulation that is modified based on activity in spinal motor neurons. In the absence of motor activity, smooth optic flow is delivered, but this stimulus is slowed or reversed when motor neurons are active **(i)**. The virtual strength of the tail can be changed while whole-brain calcium imaging is performed. **(j)** The schematic

horizontal optical flow, regularly executing rapid saccades in the opposite direction. Whole-brain calcium imaging during OKR performance has revealed the neural correlates of both the sensory and the motor elements of the behavior [38,46]. In the context of predatory behavior, the two eyes converge on the forward visual field immediately before a strike, and this convergence also occurs in response to fictive prey in an immobilized preparation. The fact that this is a rare movement, occurring only during predation, aided in linking it to specific patterns of neural activity in small assemblies of tectal neurons [26,33•].

In natural settings, larvae experience a constant interplay between stimuli and behavioral responses. The brain's functional circuitry, having evolved under these conditions, is not always well gauged in experiments where stimuli and behaviors take place in isolation. This again highlights the limitations of functional imaging in an immobilized preparation, where the larva cannot behave freely. Although calcium imaging in free-swimming larvae has been demonstrated previously, including using a bioluminescence technique not requiring optical excitation light [47], until very recently it has lacked cellular resolution [48] and been limited to moments when the larva is motionless [49,50]. The establishment this year, by a pair of groups [51••,52••], of microscopes capable of tracking free-swimming larvae while simultaneously performing volumetric imaging at cellular or near-cellular resolution is therefore a welcome and exciting development (Figure 1j,k).

An alternative to free-swimming preparations can be found in closed-loop paradigms, in which behavioral responses like movements of the tail or eyes feed back to influence the (typically visual) stimuli presented to the animal [33•,53,54,55•,56,57] (Figure 1h,i). In theory, this provides a life-like stimulus–response relationship in an immobile preparation. Recently, Naumann and colleagues [58•] used a closed-loop approach to dissect, at the behavioral level, the sensorimotor transformation underpinning the optomotor response (OMR), during which larvae swim along with sustained visual flow. They paired this with whole brain calcium imaging in stationary larvae, thus identifying specific brain regions and patterns of activity underlying the OMR. Kawashima and colleagues [55•] demonstrated a role for the dorsal raphe nucleus in motor learning. They used a closed-loop virtual swimming environment and changed how far the larva 'swam' with a given motor command. Serotonergic neurons were found to be responsible for learning the efficacy of these virtual motions.

Closed-loop paradigms and new techniques for functional imaging in free-swimming larvae each have their advantages. Free-swimming preparations provide the ultimate closed loop, where behaviors and their effects are naturally linked. Full experimental control over certain stimuli can be challenging in free-swimming larvae, however, and the current technologies for the targeted illumination of neurons (described in the next section) may not be able to keep up with a moving target (depending on the spatial resolution required). Both of these concerns are alleviated in a stationary closed-loop preparation, but it always has to be carefully assessed whether the engineered pairing of behavior and stimulus is sufficiently realistic to reveal the true nature of sensorimotor behavior.

Assessing causation in the context of whole-brain imaging

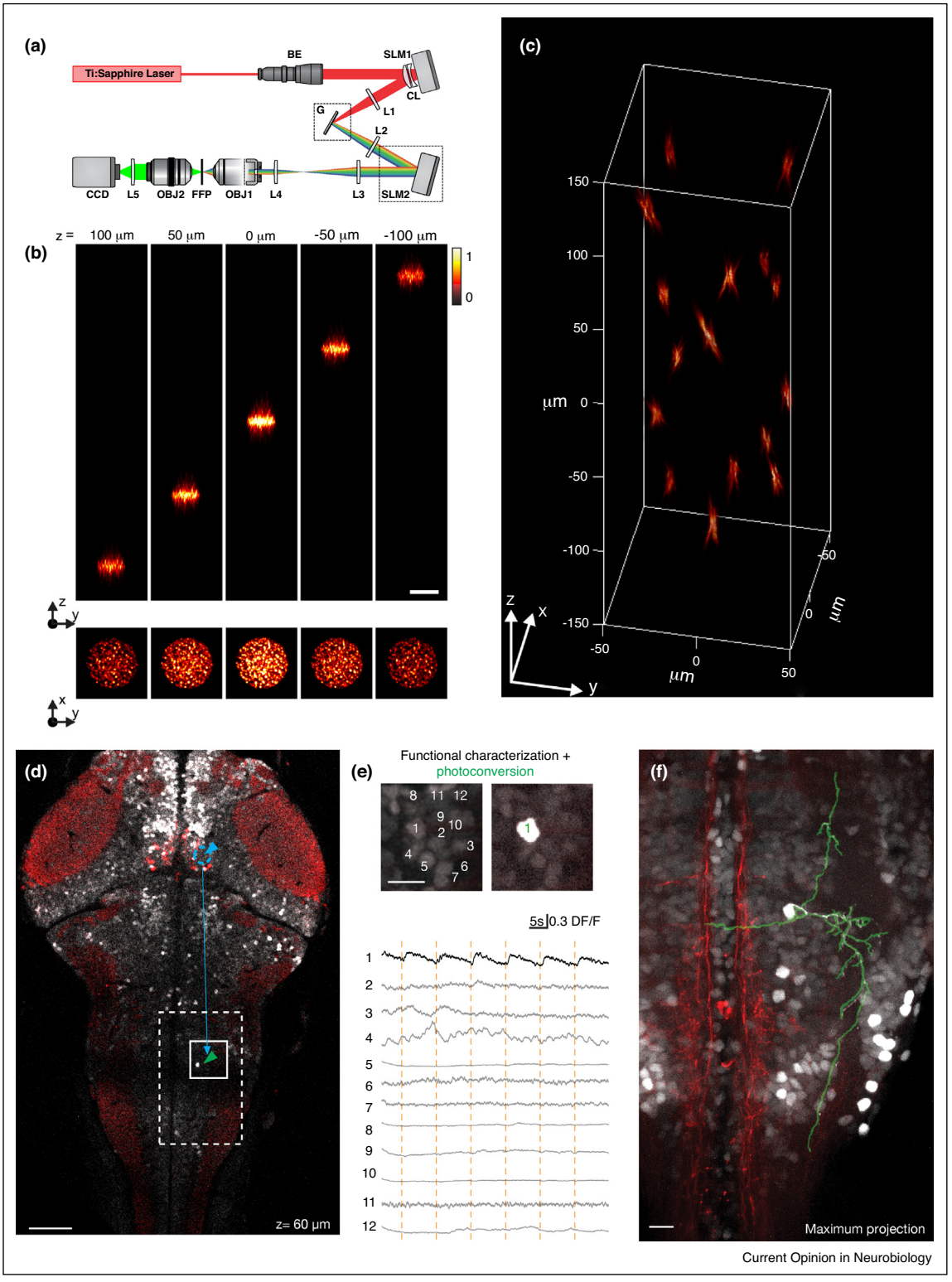
A majority of the work described above seeks to link patterns of activity in the brain to stimuli or behaviors with which they coincide. The advent of whole-brain functional imaging with cellular resolution makes this a powerful approach, as it provides a complete picture of responses found in neurons throughout the brain. This approach does not, however, reveal causal relationships among active cells, and as such, has limited utility in elucidating the underlying circuits. Instead, it provides an authoritative departure point for studies of the circuits that these neurons form, and the pathways through which the observed activity travels.

A decade ago, optogenetics emerged with the promise of elucidating the necessary and sufficient circuitry (through targeted silencing and activation, respectively) for behaviors of interest. Although optogenetic manipulations have been used in zebrafish circuit studies [42,59,60••,61–63], ablations and genetically encoded toxins also remain workhorses for assessing causality in neural circuits [35,40,58•,64,65].

The information drawn from observing or manipulating activity across populations of neurons provides clear indications of how information might flow through the brain, but this can only be placed into a convincing circuit-scale model if the structure, connectivity, and neurotransmitter type of the neurons are also known. These types of data become available if driver lines for bipartite systems like Gal4/UAS exist that are specific to neurons of interest. In

(Figure 1 Legend Continued) configuration for whole-brain calcium imaging in free-swimming larvae. Predictive code and automated 3D tracking keep the animal in frame and in focus during imaging. **(k)** Shows the path of a free-swimming larva, with whole-brain images taken from different time points of the sequence. (a–c) Show unpublished data from the Scott lab, (d–g) are modified from [44•], (h and i) are modified from [55•], and (j and k) are modified from [52••]. Scale bars are 2 mm in (b) and 100 μm in (c), and 200 μm in (d).

Figure 2



Light sculpting, optogenetics, and morphological techniques to complement whole-brain imaging. By combining two spatial light modulators (SLMs) with a galvo mirror (G), and other optical elements **(a)**, Hernandez *et al*. [76•] illuminated disc-shaped regions at different depths of a

these cases, Gal4 drivers for particular types of neurons can be crossed separately to UAS-linked activity probes and anatomical markers, and their functional and morphological characteristics can be assessed in parallel. The approach's incisiveness is eroded in zebrafish, however, by the breadth of expression that many Gal4 transgenes, especially enhancer trap lines, show. In contrast to the lines produced in invertebrates such as *Caenorhabditis elegans* and *Drosophila*, zebrafish transgenics generally drive expression in multiple brain regions and cell types [66–71], likely as a result of more complex vertebrate enhancers. Nonetheless, this approach has been used to reveal the functional characteristics of anatomically delineated neurons in the tectum [72,73] and spinal cord [63], among other regions. Along the same lines, genetic markers or immunostaining of neurotransmitter subtypes can be combined with functional imaging, allowing neurons' functional profiles to be linked to their neurotransmitter use [35,55•,73]. A set of neurotransmitter subtype-specific and other targeted Gal4 lines, recently introduced by Förster and colleagues [74•], should facilitate this approach. These lines will also, when crossed with UAS-linked activity indicators, will allow brain-wide but neurotransmitter-specific activity to be observed.

Targeted, spatially restricted manipulations are also possible through optogenetics if the activating or silencing light can be restricted to small volumes corresponding to the neurons in question, and light sculpting using spatial light modulators (SLMs) can deliver light to neuron-scale volumes deep within live zebrafish larvae [75]. In an innovative extension of this principle using a pair of SLMs, Hernandez *et al.* [76•] recently achieved the targeted illumination of numerous pre-specified neurons at different depths of the zebrafish larval spinal cord, raising the prospect of targeted circuit-scale manipulations of activity (Figure 2a–c). This result highlights the capabilities that are emerging as a result of rapid recent advances in optical physics, and how well suited these technologies are to the zebrafish model.

Putting it all together: studies that combine activity, function, and anatomy

As outlined above, whole-brain functional imaging provides a new and powerful perspective on the zebrafish nervous system, but relies on complementary anatomical, optical, and genetic techniques to deliver circuit-level insights. In a recent paper that demonstrates the power of this combined approach, Dunn *et al.* [60••] studied sequences of spontaneous turns that are thought to improve exploration in the absence of salient cues. They combined functional imaging with rapid targeting of neurons for photostimulation, photoactivation, or ablation. These tools allowed them to show the anterior rhombencephalic turning region (ARTR) was central to this behavior.

Using a different approach, Dal Maschio *et al.* [77••] (Figure 2d–f) have developed an integrated platform to probe neurons' activity while characterizing their morphology and connectivity. They combined these distinct types of information for neurons in the nucleus of the medial longitudinal fascicle (nMLF) to reconstruct the neuron-by-neuron contributions that control tail bending.

Whole-brain electron microscopy is now feasible in zebrafish, and can be registered against optical measurements of neuronal activity, as shown by Hildebrand *et al.* [78•]. This approach places active neurons in the context of a complete brain projectome, and in principle, could be linked in the future to complete connectomes. This represents an important fusion of activity with fine-grained anatomy, but the approach is unlikely to become routine in the near future, given the need to map the anatomy, using EM, in each animal studied.

Brainwide mapping of cellular properties such as neuromodulator phenotype has been combined with brainwide neuronal activity measurements during behavior by Lovett-Barron *et al.* [79•]. Activity correlates of alertness were first mapped through two-photon imaging during behavior, after which the brain was fixed, and cell types across the brain were assessed through immunohistochemistry. Registering the activity to the anatomy allowed Lovett-Barron *et al.* to identify neuromodulatory loci across the entire brain that set alertness levels.

Whether it be single cell morphology, an EM-based wiring diagram, or information on the cells' neurochemistry, the ability to combine functional imaging data with other pertinent biological information in individual animals yields datasets disproportionately more powerful than those derived from studying function and anatomy separately.

Conclusions

In the few years since the first whole-brain calcium imaging studies in zebrafish larvae, technological progress has continued apace. *In vivo* holographic light sculpting has emerged, promising experimental control over pre-specified neurons throughout large volumes of the brain, and thus bringing functional analyses of sparsely

(Figure 2 Legend Continued) homogeneous medium **(b)** and neuron-scale volumes in intact brain tissue **(c)**. Dal Maschio *et al.* [77••] used optogenetic stimulation of neurons in the nucleus of the medial longitudinal fasciculus (top **(d)**) and calcium imaging to identify a responsive downstream neuron in the hindbrain (bottom of d and cell 1 in panel **(e)**). Subsequent targeted photoconversion of this neuron revealed its morphology **(f)**. (a–c) are modified from [76•] and (d–f) are modified from [77••]. Scale bars are 50 μm in (d) and 10 μm in (f).

distributed circuits within reach. Imaging technologies that can be applied to free-swimming larvae are making it possible to view the functioning brain in naturalistic settings. Increasingly, data from whole-brain imaging are being combined with neurochemical and anatomical information to put activity into a more relevant biological context. By all indications, we are at the dawn of an astounding period of discovery into the circuit-level mechanisms underlying perception and behavior.

As hurdles to imaging this activity fall, the task of processing and interpreting these vast data become more daunting [80,81]. The storage requirements for whole-brain datasets mean that sharing these data is challenging. The connectomics community and others have attempted to meet similar challenges with online visualization and annotation of terabyte-scale datasets such as KNOSSOS [82], Neurodata (https://neurodata.io), and Cytomine [83]. As more groups gain the ability to collect these data, there is also an increasing incentive to standardize data collection and annotation so that data from different groups can be registered, or at least compared, against each other. Efforts are already underway to bridge some of the brain atlases used in the community [84,85], and these may offer the prospect of a common framework on which to register results across groups. Ultimately, a goal would be to combine the different modalities of information, from whole-brain activity maps to EM datasets, into a searchable database of neuronal types and morphologies, along the lines of searchable databases that already exist for gross neuronal morphology [86,87].

Having a reproducible analysis workflow and repositories for data sharing across the zebrafish neuroscience community would expand such comparisons, as well as allow exploratory analysis of these datasets beyond what individual groups are able to do. Open science efforts, such as the Allen Institute for Brain Science, have shown the benefits that data sharing can have in attaining and integrating new knowledge, as well as improving the reliability of our interpretations. One could imagine such an international consortium of zebrafish neuroscientists coming together to share and integrate their data in an effort toward a holistic functional model of the larval zebrafish brain.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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Favre-Bulle and colleagues used optical trapping, a method for applying force with a focused laser, to move otoliths in the inner ear of larval zebrafish. This allowed the controlled stimulation of the vestibular system, without the stimulation of other modalities, in an immobile animal. These simulated lateral motions triggered corrective motions of both eyes and tail, and induced forward swim bouts when strong forces were applied.

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Using a light-field approach, Cong *et al*. offer higher speeds than the Kim *et al*. implementation, at up to 77 volumes per second. This may be useful if faster probes are developed or when using spike deconvolution. The signal to noise ratio of LFM is also higher than structed illumination. Resolution is lower, however, and the lack of predictive tracking may hinder its efficiency in fast high-speed behaviors.

52. •• Kim DH, Kim J, Marques JC, Grama A, Hildebrand DGC, Gu W, Li JM, Robson DN: **Pan-neuronal calcium imaging with cellular resolution in freely swimming zebrafish**. *Nat Meth* 2017, **14**:1107-1114.

Kim *et al*.'s differential illumination focal filtering allows reasonably fast volumetric acquisition (2 volumes per second) of freely swimming zebrafish larvae at high resolution. The signal to noise ratio is low, however, and

may require averaging across several trials. Constraints on movement in the *z*-axis may still limit some behaviors, but this approach nonetheless opens new options for pairing behavioral studies with whole-brain imaging in zebrafish.

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54. Jouary A, Haudrechy M, Candelier R, Sumbre G: **A 2D virtual reality system for visual goal-driven navigation in zebrafish larvae**. *Sci Rep* 2016, **6**:34015.

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The serotonergic neurons of the dorsal raphe nucleus were found to persistently encode the efficacy of locomotor drive. This allowed the larvae to adjust the gain of motor commands in a closed-loop virtual environment. Ablation of the serotonergic neurons or blocking of serotonin release stopped this learning, while optogenetic activation lowered the locomotor drive of subsequent virtual swims. Using these results, they built a model of the DRN neurons as slow leaky integrators that attenuate the motor commands.

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Naumann *et al*. used a closed-loop preparation to dissect the transformation of the optical stimuli to motor commands. Once the locomotion patterns were defined, they used whole-brain calcium imaging to identify their neural substrates. In the end, they built a beautiful model of the circuit underlying the sensorimotor transformation of the OMR.

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Dunn et al. identified the anterior rhombencephalic turning region (ARTR) as a necessary and sufficient driver of spontaneous turn bias. They were able to activate or ablate specific neurons in the ARTR, these perturbations biased the sequence of turns. Using PA-GFP, they further traced the ARTR neurons to the inferior olive, nucleus of the medial longitudinal fasciculus (nMLF) and reticulospinal neurons. By combining these streams of evidence, they were able to build a convincing model of the circuit that drives exploratory behaviors.

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Förster *et al*. have generated 22 transgenic zebrafish lines that employ the Gal4/UAS or Cre/lox systems. Using BAC recombineering to assure faithful recapitulation of selected genes' expression patterns, they have created lines with drivers restricted to neurons expressing specific transcription factors or neuropeptides, or using specific neurotransmitters. These lines should allow analyses or optogenetic manipulations specifically in these expression patterns, or at the intersections of multiple patterns.

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Two SLMs set up in sequence allowed Hernandez and colleagues to uncouple the axial and lateral shaping of light projected into the brain. They produced multiple spatially restricted shapes with precise parameters, which allowed them to photoconvert multiple cells at various depths at once. This method could be used to photoactive multiple neurons in parallel.

77. •• Dal Maschio M, Donovan JC, Helmbrecht TO, Baier H: **Linking neurons to network function and behavior by two-photon holographic optogenetics and volumetric imaging**. *Neuron* 2017, **94**:774-789 e775.

Dal Maschio *et al*. combine targeted photostimulation of neurons, using holography, with behavior tracking and whole brain imaging. The same approach was used to reconstruct the morphology of identified neurons using PA-GFP. These methods allowed them to dissect the circuits of the nMLF involved in tail bending or swimming. To avoid the trap of combinatorial explosion, they used a clever iterative approach to restrict the space of possibilities and could pinpoint the contribution individual neurons made to the behavior.

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Hildebrandt *et al*. built a beautiful multi-scale reconstruction of the zebrafish brain using serial-sectioning and electron microscopy. They

then registered GCaMP fluorescence imaging to the EM results. This dataset was also registered to the Z-brain atlas, linking functional information to the anatomy. Being able to trace neurons and looking at their connections will increase the power of whole-brain imaging observations.

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Lovett-Barron *et al*. developed the multiplexed alignment of molecular and activity phenotypes (Multi-MAP) approach to combine antibody labels with live calcium imaging using whole-brain registration. They could further leverage the power of their approach by registering the results to the Z-brain atlas and take advantage of its brain region delineations. With this tool they identified 22 neuromodulatory cell types and characterized their response profile before and after looming stimuli to investigate their role in alertness.

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