Tracking microscopy enables whole-brain imaging in freely moving zebrafish

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Efficient tracking and optical whole-brain imaging at single-cell resolution in freely behaving zebrafish larvae pave the way for quantitative investigation of circuits underlying complex behaviors.

One of the main goals of modern neuroscience is to determine how the coordinated activity of distributed neuronal networks underlies complex behaviors when an individual interacts with its environment. Three studies published in the current issues of *Nature Methods*[1,2] and *eLife*[3] report technological breakthroughs that enable monitoring of brain activity with high spatial resolution in a freely behaving vertebrate.

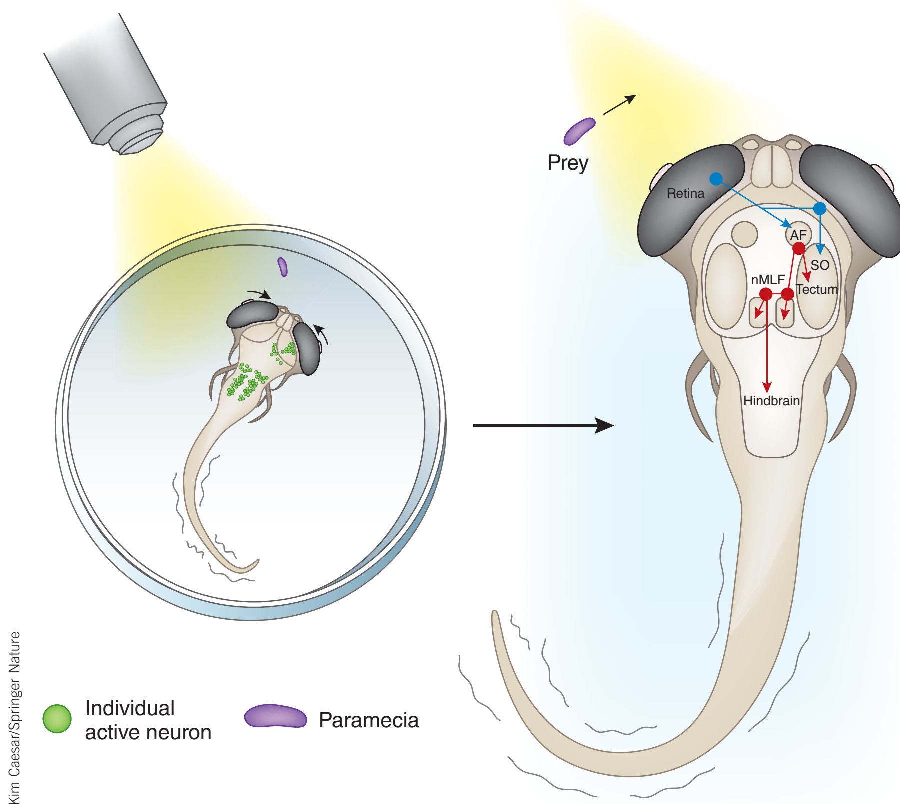

Figure 1 | Imaging whole-brain neuronal activity at single-cell resolution is now possible in freely moving zebrafish larvae performing complex sensory–motor tasks. The technology will help establish the neuronal networks being recruited during these tasks, as is depicted here for the prey-capture circuit.

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Classical approaches to achieving brain-activity mapping in humans have relied on electroencephalography and magnetoencephalography or functional magnetic resonance imaging. Such techniques are limited to recording global brain activity, and they typically integrate the activity of several thousand neurons. In contrast, optical imaging of activity-dependent fluorescent sensors can resolve single neurons in small genetic model organisms, such as the worm *Caenorhabditis elegans* or the zebrafish larva. This worm's small size and low speed enabled researchers to combine real-time tracking of its head with a fast translation stage while performing 3D calcium imaging of its brain neuronal activity[4]. The zebrafish larva has the advantage that it is a transparent vertebrate model organism with ~100,000 neurons contained in a small volume of ~500-μm diameter. Recent implementations of light-sheet microscopy[5,6] have enabled the recording of whole-brain activity with 3D resolution around $0.5 \times 0.5 \times 5\ \mu m^3$. Yet, in these studies, the animal's head was embedded in agarose, and only its tail could move. Under these conditions, zebrafish larvae's responses to sensory stimuli, acoustic escape responses or prey capture are altered[7].

Previous attempts to estimate neuronal activity in freely moving larvae were limited to bioluminescence recordings where the location of active neurons could not be resolved[7]. Symvoulidis *et al.*[1] report a first step toward imaging active brain regions in animals freely swimming across a behavioral arena. The researchers implemented widefield microscopy to reach imaging speed above 50 images per second with a scanning field adjusted to the position of the larva, which was tracked with an infrared camera. The use of a tunable lens enabled them to adapt both magnification and axial positioning. The setup is relatively simple and runs on custom-made, open-source software. However, on account of the low spatial resolution (~10 μm) and the absence of optical sectioning, this technique cannot resolve all neurons in the brain. Nonetheless, this approach shows a net improvement over

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bioluminescence recording, as it allows the identification of narrow active brain regions involved in a diverse set of behaviors.

To image the brain of swimming larval zebrafish at single-cell resolution, it is necessary to cope with the extreme peak acceleration of the animal (20 m s^{-2}). A solution to this problem is to couple a fast tracking system with a fast optical technique that is sensitive enough to detect activity-dependent changes of fluorescence in spatially resolved neurons within submillisecond time scales. Kim *et al.*[2] achieved this impressive task by performing calcium imaging in freely swimming larval zebrafish using high-speed tracking microscopy. The authors implemented 3D monitoring of the whole brain combined with a custom-made real-time tracking algorithm that predicts the future displacement of the larva based on modeling its locomotion from its current position as monitored with widefield infrared imaging. The brain-imaging technique, a variant of HiLo microscopy[8], requires only two images per optical section, increases contrast and has a spatial resolution of ~$1 \times 1 \times 4.4$ μm^3 per plane. The authors monitored brain activity at two volumes per second in freely swimming animals. In addition, the authors developed and characterized a custom gPU-based registration pipeline to eliminate motion artifacts caused by variable animal posture and deformation of nonrigid tissue. The tracking microscope enables continuous whole-brain imaging of neural activity at single-cell resolution across hour-long imaging experiments.

Cong *et al.*[3] also report fast 3D recording of neurons in the freely swimming zebrafish larva brain. The authors used a variant of light-field microscopy[9,10] that permitted fast imaging of the entire brain volume in a single camera frame at ~75 volumes per second. Compared to usual light-field microscopy, the authors report an improvement of resolution down to ~$3.4 \times 3.4 \times 5$ μm^3 over a large imaging volume of up to $500 \times 500 \times 200$ μm^3. This imaging resolution can only resolve single neurons if the density of simultaneously active cells is low (around 10% of all neurons). The authors combined this imaging technique with a conventional three-axis moving stage to track the larva. Some applications, such as the investigation of motor circuits enabling left–right alternation in the hindbrain during locomotion, will benefit from such fast volume rates.

In the future, light-field microscopy could be advantageously coupled with the tracking system developed by Kim *et al.*[2]. Such a combination of smart tracking[2] and fast volume imaging[3] will further improve the investigation of circuits underlying complex behaviors of zebrafish larvae, such as capture of real prey, spatial preference and learning assays, as illustrated in **Figure 1**. Furthermore, increasing imaging depth by combining two-photon excitation and wavefront shaping[11] with tracking microscopy may bring new perspectives to investigate complex and mature behaviors such as fear response or social preference in the large brain of juveniles and adults. Combining these techniques with optogenetics will enable the interrogation of neural network activity underlying complex behaviors. Ultimately, the complete reconstruction and probing of neuronal network dynamics underlying naturalistic behavior will make it possible for researchers to test realistic models that underlie decision making. And, beyond neuroscience, imaging at single-cell resolution in freely moving zebrafish larva will benefit investigations into cellular dynamics within diverse organs in living vertebrates.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Super-resolution imaging goes fast and deep

Sam Duwé & Peter Dedecker

Advances in image scanning microscopy move super-resolution imaging deeper into tissues with faster visualization and finer details.

Fluorescence microscopy is the imaging technique of choice for many researchers, and even more so with the advent of super-resolution imaging. While these super-resolution techniques can offer a dramatically increased spatial resolution, they do come with various limitations, such as slower measurements and higher light doses. Over the past few years, work by several research groups has resulted in the development of image scanning microscopy (ISM), a method that increases the spatial resolution of confocal instruments by up to a factor of two without fundamentally reducing their performance or applicability. Now, two developments reported by the laboratories of Shroff[1] and Enderlein[2] dramatically enhance ISM performance in two-photon excitation (2PE) microscopy, resulting in deeper and faster imaging, while they also expand the use of ISM to second harmonic generation (SHG) microscopy.

It has long been known that adding a very small pinhole to an optical microscope can improve its spatial resolution up to two-fold. Unfortunately, this addition also decimates the detectable signal, so the pinholes in actual confocal instruments are much larger, which sacrifices most of the resolution improvement. ISM enables researchers to use an optical microscope as if it had a tiny pinhole, and thus it improves the resolution two-fold without compromising sensitivity or imaging speed[3].

The key to ISM is to replace the single pinhole and detector with a grid of detectors, each of which acts like a tiny pinhole. While one such detector detects considerably less signal, there is a net gain in detected signal in ISM because the grid covers the entire emission spot. Most of these detector pixels are located away from the center of focus (the optical axis), and they collect fluorescence from a sample region that is likewise shifted from the optical axis (**Fig. 1a**). This is where a key aspect of ISM comes in: even though the detector in **Figure 1a** can in principle record emission for the full region highlighted by the green curve, this fluorescence can only arise

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