COMMENTARY

Grabbing brain activity on the go

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Animal behavior emerges from the dynamics of brain activity. Without a method to simultaneously measure both neural responses and behavior, inferences about the relationship between the two must necessarily remain indirect. Here Nguyen et al. (1) and Venkatachalam et al. (2) close this fundamental gap by developing techniques for monitoring neural activity with cellular resolution in freely moving *Caenorhabditis elegans* nematodes.

These and other recent studies (3–5) exploit the recent development of sensitive, bright genetically encoded calcium indicators (6), a rapid imaging modality that affords high-resolution optical sectioning, and the worm's transparent, compact body and nervous system (7). In this context, the term "compact" refers not only to the small number of neurons, namely 302, but also to their physical association: ~60% of neuronal cell bodies are contained within neuronal ganglia encompassing a cylinder that is ~40 μm in diameter and ~120 μm in length, estimated from the number of neuronal cell bodies between the nose and the anterior border of the retrovesicular ganglion (8,*) and the dimensions of a young adult animal. To image this single field of view at high speed, these groups, as well as a previous study (3), customized spinning disk confocal microscopy systems to allow high-resolution volumetric imaging at 6–10 Hz. To cope with moving animals, the current studies used closed loop feedback between worm movement and a motorized stage to keep the animal's head in the field of view.

In technical tours-de-force, Nguyen et al. (1) and Venkatachalam et al. (2) achieved parallel imaging of as many as 80 single neuronal cell bodies with behavioral tracking. Both studies express the genetically encoded calcium sensor, GCaMP6s, and a calcium-insensitive fluorescent protein, TagRFP, under the control of pan-neuronal promoters and report ratiometric changes in fluorescence. This ratiometric strategy reduces motion-induced noise, as demonstrated analytically (1). Whereas Nguyen et al. engineered animals to express both the calcium sensor and the anatomical tagRFP marker only in nuclei, Venkatachalam et al. targeted only the tagRFP marker to nuclei, while monitoring calcium signals in the cytosol (Fig. 1). The two strategies impose different technical challenges. In the latter situation, the green calcium-dependent fluorescent signal overlaps with the red, calcium-independent signal, and the activity-related signals are from the cytoplasm, but the boundaries between cell bodies are indistinct, making segmentation harder. In the former situation, there is a one-to-one correspondence between the green, calcium-dependent signal and the red, calcium-independent signal, and separate nuclei belonging to distinct neurons are easily distinguished, but calcium-dependent signals are derived from the nucleus. Fortunately, nuclear and cytoplasmic calcium signals in *C. elegans* neurons appear to differ primarily in their kinetics (3). Additionally, studies in mammalian neurons show that nuclear calcium signals follow action potentials (9). Together, these findings lessen concern regarding the use of nuclear calcium signals as a proxy for neuronal activity.

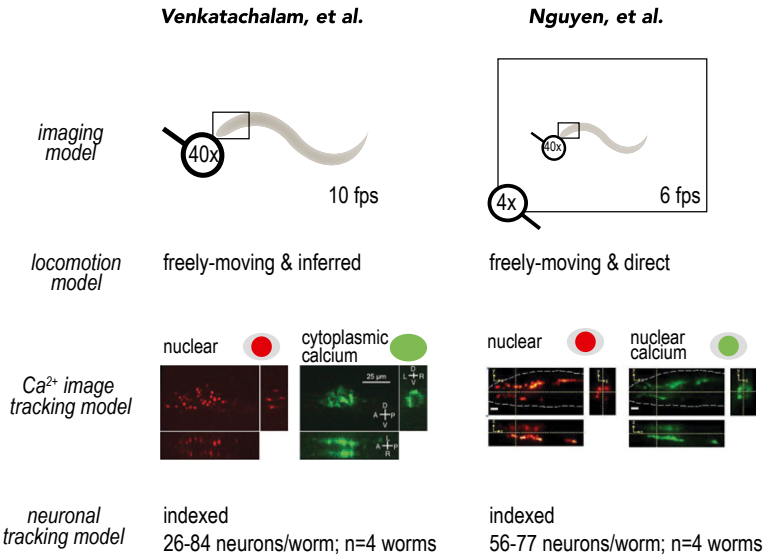

Fig. 1. A comparison of strategies for imaging neurons on the go. Photomicrographs reproduced from refs. 2 (*Left*) and 1 (*Right*).

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*www.wormatlas.org/neuronalwiring.html.

Nguyen et al. (1) and Venkatachalam et al. (2) report divergent approaches to tracking worm movement during imaging. In the latter case (2), the worm's head was kept within the imaging volume by a feedback-controlled, motorized stage, and the worm's motion was inferred from the orientation of the head. In principle, the accuracy of this inferred motion model depends on the finding that the worm's body follows the trajectory of the head (10). The inferred locomotion algorithm was assessed by comparing inferred to measured locomotion trajectories disseminated in a database of single-worm tracking studies (11). In the former case (1), animal movements were recorded by a second, low-magnification objective, and motion was measured directly. Both the inferred and direct motion models yield information about animal movement in conjunction with neural activity and work well. The direct motion approach may enable future studies of other parts of the nervous system, whereas the inferred motion strategy is compatible with experimental designs involving opaque substrates.

A major goal of all techniques for whole-brain imaging at single-neuron resolution is to discover how neural activity patterns are connected to behavior and modulated by sensory input. Previous studies connected the trajectories of freely moving *C. elegans* to states of motion—animals are said to move forward, turn, or reverse direction (12–16). Forward and backward movements are thought to depend on specific command interneurons, AVB (forward) and AVA (reverse) (17). Are there other neurons whose activities are correlated with movement state? The answer appears to be "yes": Both studies show that the *C. elegans* nervous system has distinct clusters of neurons whose activity increases during forward movement and anticorrelated clusters whose activity increases during backward movement (1, 2). Based on a principal component analysis of one data set, Venkatachalam et al. further infer that a large fraction of the neurons in the imaged volume appear to carry signals closely allied to locomotion. Similarly, half of the imaged neurons analyzed by Nguyen et al. were correlated with either forward or reverse locomotion. However, the fraction of movement-linked neurons varied considerably among the four animals imaged during movement. Thus, significant uncertainties remain about the size and extent of movement-linked clusters, and it is unclear how stable or stereotypic the activity patterns of these movement-linked neural clusters are across behavioral states and between individuals. Nonetheless, these findings, as well as those of Kato et al. (3), support the notion that a significant portion of the brain is devoted to encoding information about its behavioral outputs.

How do the patterns of neural activity measured by Nguyen et al. and Venkatachalam et al. map onto the identities of specific cells? Although it is possible to index and analyze *ca.* 50–80 neurons within a given image stack, movement-induced distortions and variation in cell body position complicate efforts to position the indexed neurons within the worm's neuroanatomical atlas (1, 2). Thus, a major challenge lies in devising strategies that allow for the neuronal cell bodies within the imaging volume to be uniquely identified. Hybrid strategies combining position and activity patterns can yield provisional identifications (3), but they rely on assumptions about the nature of the expected activity derived from prior or parallel analysis of each class of neuron. Ambiguities may complicate such hybrid strategies if neuronal activity patterns depend on longer-lived behavioral or neuromodulatory states. On the other hand, a combinatorial strategy for neuronal identification via nuclear-localized fluorescent proteins might overcome some of these limitations and likely also enable improvements in algorithms for combined neuronal and behavioral activity tracking.

A corollary of the large movement-linked clusters is that neurons whose activity is primarily driven by sensory stimulation may be rare. Consistent with this idea, Venkatachalam et al. (2) detected a single pair of neurons whose activity was modulated by sinusoidal variations in substrate temperature. As expected, the thermo sensory pair are the AFD neurons known from previous studies to respond to thermal fluctuations (18, 19). This finding suggests that sensory information is sparsely represented in the worm's nervous system. However, this inference may be premature, because fewer than half of the neuronal cell bodies contained within the imaging volume are analyzed (50–80 out of *ca.* 180) with existing techniques, and the sensory landscape has not yet been fully explored.

Techniques for whole-brain imaging at single-neuron resolution hold a great deal of promise for linking patterns of neural activity to behavior. Such approaches also provide fascinating technical challenges. For instance, targeting calcium indicators to neuronal nuclei reduces the image segmentation problem to a matter of reliably tracking volumes of interest through time, while also imposing a low-pass temporal filter on the recorded signals (3). If such a hypothetical "nuclear filter" were invariant across neurons, then this limitation could be overcome by a global deconvolution. If the nuclear filter were to vary between neurons, however, using nuclear calcium signals as a proxy for neural activity could create spurious activity correlations or obscure true correlations. The nature of the relationship between cytoplasmic and nuclear calcium signals and its variation among neurons is not currently known.

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Signals derived from identified neurons expressing GCaMP6s in nuclei or in the cytoplasm of *C. elegans* neurons support the idea that nuclear signals are slow compared with those in the cytoplasm (3), but it remains possible that they are also transformed in other ways in some cells. In principle, a direct comparison would be possible by coexpressing red and green fluorescent indicators and directing one indicator to the cytoplasm (with a nuclear export signal) and one to the nucleus (with a nuclear localization signal). Such a measurement would reveal the limits of nuclear calcium as a proxy for neural activity. More broadly, reliance on cytoplasmic or nuclear calcium signals in cell bodies makes local signaling in neurites invisible, hiding the very calcium signal likely to be most relevant to synaptic transmission. This gap in our ability to measure neural activity won't prevent us from extracting considerable insight from whole-brain imaging, but it may cause us to underestimate some important correlations as the landscape of neural network interactions is explored.

The concurrent analysis of single-neuron activity and animal behavior represents a significant step forward that will undoubtedly inspire new lines of investigation into how neural circuits control locomotion and are influenced by sensory input. Venkatachalam et al. (2) demonstrate that their system can be generalized to monitor neural activity in the fruit fly larva, paralleling efforts that have imaged neural activity in all or part of the brain in immobilized zebrafish (20), larval and adult fruit flies (21, 22), and mice (23). Most of these latter approaches deployed fictive motion, virtual reality, or other situations in which animals

need not move during imaging. Nguyen et al. (1) and Venkatachalam et al. (2) now show that, at least for the worm, "head fixed" is no longer the rule. By directly closing the loop between brain and behavior and by the future introduction of real-time perturbations of neural activity, these studies establish a strong foundation for dissecting the mind of the worm.

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