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Imaging whole-brain activity to understand behavior

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Abstract

The brain evolved to produce behaviors that help an animal inhabit the natural world. During natural behaviors, the brain is engaged in many levels of activity from the detection of sensory inputs to decision-making to motor planning and execution. To date, most brain studies have focused on small numbers of neurons that interact in limited circuits. This allows analyzing individual computations or steps of neural processing. During behavior, however, brain activity must integrate multiple circuits in different brain regions. The activities of different brain regions are not isolated, but may be contingent on one another. Coordinated and concurrent activity within and across brain areas is organized by (1) sensory information from the environment, (2) the animal's internal behavioral state, and (3) recurrent networks of synaptic and non-synaptic connectivity. Whole-brain recording with cellular resolution provides a new opportunity to dissect the neural basis of behavior, but whole-brain activity is also mutually contingent on behavior itself. This is especially true for natural behaviors like navigation, mating, or hunting, which require dynamic interaction between the animal, its environment, and other animals. In such behaviors, the sensory experience of an unrestrained animal is actively shaped by its movements and decisions. Many of the signaling and feedback pathways that an animal uses to guide behavior only occur in freely moving animals. Recent technological advances have enabled whole-brain recording in small behaving animals including nematodes, flies, and zebrafish. These whole-brain experiments capture neural activity with cellular resolution spanning sensory, decision-making, and motor circuits, and thereby demand new theoretical approaches that integrate brain dynamics with behavioral dynamics. Here, we review the experimental and theoretical methods that are being employed to understand animal behavior and whole-brain activity, and the opportunities for physics to contribute to this emerging field of systems neuroscience.

Introduction

Animals possess repertoires of natural behaviors that allow them to navigate the world, interact with the environment, and interact with other animals. Examples include searching for mates, hunting prey, or escaping from predators. These behaviors require animals to simultaneously process many different sensory experiences, make different types of decisions on multiple timescales, and continuously monitor and modify their own movements and behavioral performance. Natural behaviors are not easily reduced to one-to-one mappings from sensory stimulus to motor output, as can be done for feedforward reflexes. Instead, natural behaviors engage many types of neural computation at the same time — multisensory processing, memory storage and recall, decision-making, motor production, feedback, and control mechanisms — in ways that cannot be compartmentalized. These computations are often carried out by many brain areas acting together, communicating via system-wide networks of synaptic connectivity and non-synaptic modulation.

To understand the relationship between whole-brain activity and behavior, we turn to animals where it is possible to access the entire brain during behavior with minimal artificial constraints. Only a few model organisms permit whole-brain activity recording in intact animals during natural behaviors. Here, we focus on the nematode *Caenorhabditis elegans,* the fruit fly *Drosophila melanogaster,* and the larval zebrafish *Danio rerio* (Box 1). Other small animals like the hydra are being developed as models for whole-brain and whole-circuit approaches to behavior[1]. There has also been much recent work in rodents where large numbers of neurons can be recorded in rich behavioral contexts[2-5]. These systems allow circuit-level dissections of behavior[6;7]. However, it is not yet possible to record from whole mammalian brains with full cellular resolution. The development of neuropixel electrodes has increased the throughput of electrophysiological brain recordings in mammals, but without the full field-of-view and resolution of microscopy systems[8]. Functional magnetic resonance imaging (fMRI) relies on changes in bloodflow to different brain regions to visualize whole-brain activity, but has low spatial resolution when compared to optical methods and is not a direct measurement of neuron activity[9;10]. We thus limit this review to discussing imaging approaches to whole-brain activity in behaving animals.

The capacity to comprehensively record the brains of worms, flies, and fish during behavior arose with recent developments in microscopy. Fast, high-throughput microscopes combine rapid volumetric imaging with three-dimensional tracking of brain-wide dynamics. Many of these imaging systems are also capable of simultaneously monitoring the behavioral dynamics in unrestrained, semi-restrained, or virtual reality experimental setups. Advances in imaging technology and data analysis will continue to expand the range of possible experiments, allowing the acquisition of complete brain recordings during more types of behavior[11].

Systems neuroscience in worms, flies, and fish is now generating rich datasets of brain-wide activity that span multiple sensory inputs, distributed circuits, and different behaviors. Understanding these datasets requires innovation in theory and computation. Do we understand the brain when we fully map the detailed patterns of co-variation between

sensory inputs, brain activity, and motor responses? Are there principles of integrated brain function that impose low-dimensional structure on the correlations between sensation, cognition, and action? How does anatomical wiring impose constraints that can be used to better understand brain and behavioral dynamics?

Here, we review (1) the technological advances that have enabled rich recordings of whole-brain activity and behavior, (2) recent experiments in model organisms that have captured behaviorally-relevant brain-wide activity, (3) and computational and theoretical approaches that attempt to link brain activity to behavior. At each stage, we highlight ways in which physicists have contributed to this exciting field and the many opportunities for future work.

Experimental methods for whole-brain imaging

On time scales shorter than a second and spatial scales longer than hundreds of microns, diffusion is too slow to synchronize cellular or system-wide activity. To rapidly coordinate the activity of sensory and motor systems over long distances, neurons rapidly propagate electrical signals along fibers throughout the nervous system. Electrical signaling is coupled to changes in the intracellular concentrations of multiple ions, including calcium. These changes are typically followed by the activation of intracellular signaling pathways and eventually cell-to-cell communication by short-range synaptic transmission and long-range neuromodulation[12]. Therefore, measuring activity at the whole-brain level requires microscopic probes that can globally detect changes in electric fields, intracellular ion concentration, or neurotransmitter release.

One of the most successful approaches has been to use microscopy to capture activity-dependent fluorescence from proteins expressed in neurons of transgenic animals. Genetically-encoded sensors derived from fluorescent proteins have been developed for many aspects of neuronal activity[13-16]. The studies we review here primarily use the GCaMP family of indicators, derived from green fluorescent protein (GFP), which have proven to be well-suited for stable, long-term imaging of large populations of neurons in many genetically accessible animals[2].

After choosing a fluorescent sensor, microscopes are needed to resolve single cells throughout the brain while sampling at informative timescales of behavioral and neuronal activity, from milliseconds to minutes or longer. The most common approach to imaging many cells with single-cell resolution using fluorescence is to confine the excitation light to a portion of the imaging volume, selectively capture in-focus light from that portion, and then serially scan the brain volume. This approach underlies confocal, two-photon (2P), structured illumination, and light-sheet microscopy[17-21].

Conventional two-photon and confocal approaches use point scanning to image a brain volume. Point scanning has advantages in optical resolution, but is typically too slow to image many cells throughout a large brain volume on subsecond timescales. Confocal microscopy can be accelerated by simultaneously scanning many points in a focal plane using a 2D array of pinholes (spinning disk confocal microscopy). Two-photon laser scanning microscopy (2PLSM) allows deeper imaging into larger brains, and can be

accelerated by adaptive, closed-loop scanning to improve image acquisition speed for behaving animals[22;23].

Living biological samples are generally more susceptible to photodamage than inanimate samples when subjected to laser light. Light-sheet microscopy confines light to an imaging plane without allowing propagation into parallel planes, allowing optical sectioning with minimal photodamage. Many light-sheet microscope setups use separate objectives for delivering excitation light and recording fluorescence, imposing physical constraints on the animal being recorded and limiting the behaviors that can be studied[24]. New single-objective light-sheet approaches permit rapid volumetric imaging with low photodamage and modest trade-offs in resolution, expanding the range of behaviors and animals that can be studied[21].

Another approach is to use optics to capture information from a three-dimensional volume directly on a two dimensional sensor, albeit at the cost of *xy*-resolution and field of view. One way to accomplish this is to tile images from different focal depths on the sensor (multifocus microscopy)[25;26]. A related strategy is light-field microscopy, which uses microlens arrays to preserve three-dimensional information in the emitted rays to enable computational reconstruction of volumes from sensor data[27-31].

Whatever the optical hardware, whole-brain imaging also requires complete optical access to every neuron inside a behaving animal. Small animals with transparent bodies and brains like nematodes, larval *Drosophila,* and larval zebrafish are natural candidates. The heads of non-transparent animals, including adult *Drosophila* and larger vertebrates, must be surgically opened to view the brain, or have microscopes inserted into the brain. The development of non-invasive strategies to image without surgery will allow cleaner access to behaviorally-relevant brain activity[32]. Expanding the toolbox of techniques for whole brain recording will increase the numbers of animals and behaviors that can be studied with systems-level approaches.

As our ability to perform whole-brain imaging during behavior increases, so does the problem of dealing with the enormous amount of data that it rapidly generates. Microscopes measuring whole-brain neuronal activity easily generate raw image data at 1 GB/s or more. These data must be reduced into compact time traces corresponding to the activity of discrete neurons or brain regions. Segregating the activity of individual neurons is challenging when neurons and nerve fibers are densely packed in a brain volume or when neurons move relative to one another because of animal self-movement. Continuously tracking neurons inside the rapidly deforming brain of a freely moving *C. elegans* or *Drosophila* larva is as challenging as acquiring the volumetric images in the first place. To complement the optical hardware that performs whole-brain imaging, image-processing algorithms that are both fast and accurate are needed to meet the challenge of comprehensive neuronal segmentation[33-37].

Whole-brain imaging in three model organisms

Box 1 describes three model organisms considered in this review: the nematode *Caenorhabditis elegans,* the fruit fly *Drosophila melanogaster,* and the larval zebrafish

Danio rerio. Whole-brain imaging methods are now being used to study these animals. These methods were first demonstrated in immobilized animals, where brain activity was correlated with fictive behavioral read-outs, such as the activity of muscles or command motor neurons. Recent breakthroughs have made it possible to extract whole-brain activity from animals behaving more naturally and navigating real or virtual spaces nearly unimpeded. We briefly review some of the unique advantages of these three animals and how whole-brain imaging has advanced our understanding of their behavior.

The nematode *C. elegans*

The compact nervous system of the nematode *C. elegans* is ideal for whole-brain experiments. Each *C. elegans* hermaphrodite has 302 neurons with a largely stereotyped wiring diagram – about 200 neurons form an anterior brain; about 100 neurons form the motor circuit[38;39]. An additional 100 sex-specific neurons in the tail of the male worm orchestrate mating behavior[40].

The worm brain's small size allows it to be rapidly imaged with single cell resolution using light microscopy – either the anterior brain shared by both sexes or the posterior male-specific "brain"[41]. Whole-brain imaging was pioneered in immobilized worms, where it was discovered that even in the absence of external stimulus, a large proportion of the brain's neurons engage in coordinated activity. When this whole-brain activity is projected onto a low-dimensional representation, brain dynamics follow a cyclical trajectory[42]. Portions of the cycle correspond to the activity of pre-motor interneurons known to be associated with locomotion direction, allowing epochs of fictive forward and backward movement to be inferred in stationary animals. The stereotyped brain-wide activity patterns for forward/backward behavioral states have been interpreted to represent global commands that account for the majority of the variance in neural dynamics.

Forward and backward locomotion are slowly changing behavioral states, but muscle activity within each state occurs on faster time scales to drive rapid exploratory head bending and rhythmic body undulation[43]. Although the neurons that drive these movements operate at much faster time scales, they are directly modulated by other neurons with slowly changing activity that are correlated with forward/backward behavioral state changes. The activity and cross-modulation of neurons across a hierarchy of time scales occurs in both moving and immobilized worms. Nested activity dynamics across time scales appears to be an organizing principle of the brain circuit, both during unrestrained and fictive behavior[44].

Comparing whole-brain dynamics in immobilized animals to separate behavioral experiments in moving animals can illuminate correlations between circuit activity and behavior. To more carefully dissect the mechanisms in whole-brain dynamics that produce behavior, brain and behavioral dynamics can be studied at the same time in the same animal. Improvements in volumetric imaging speed and single neuron tracking now enable whole-brain recording in freely moving worms[45;46]. As observed in immobilized worms, large numbers of neurons in the brain are correlated with forward and backward movement. In freely moving worms, however, substantial diversity in brain dynamics is observed, with activity often correlated with additional quantifiable parameters of worm movement such as velocity and curvature. Reliably decoding these behavioral details from brain-wide activity

requires large numbers of neurons, hinting at a more subtle and distributed neural code for the full dynamics of worm behavior[47]. Moreover, the correlation structure between certain pairs of neurons changed dramatically when freely-moving worms were immobilized. Thus, the neural dynamics of fictive behaviors in immobilized worms are measurably different from the corresponding neural dynamics in unrestrained worms, an important caveat when trying to understand a natural behavior by studying immobilized animals.

Another challenge in whole-brain recording is matching neurons between animals. In *C. elegans,* every neuron follows a stereotyped lineage across development and has a largely stereotyped connectivity to other neurons. In principle, one should be able to compare whole-brain activity of different animals by aligning the activities of the same neurons. However, animal-to-animal variability in the relative positions of cell bodies makes it the neuronal identities difficult to determine. To identify neurons, one needs additional cell-specific information. Substantial knowledge of gene expression patterns in *C. elegans* provides a means of adding identifiers to neurons. Labeling a cell or group of cells of interest with a fluorescent protein with an emission spectrum orthogonal to that of the calcium sensor allows specific cells to be tagged and identified. Recently, a combinatorial method of adding many fluorescent labels of different colors was applied to the entire nervous system, facilitating neuron identification during whole-brain imaging[48].

The tail of the male *C. elegans* contains a separate brain for mating with hermaphrodites[49]. Male mating behavior is a complex multi-step behavior composed of numerous component behaviors that occur in different stimulus-evoked sequences from event to event. The male recognizes the different parts of the hermaphrodite body as he circles around her (and as she generally tries to escape from him), and makes many different movement decisions as he searches for the vulva and copulates. The entire mating circuit in the male tail can be imaged continuously while the male performs all steps of mating behavior. The full diversity of stimulus and motor patterns that occur during mating behavior are represented in a similarly diverse set of neuronal activity patterns in the male tail. The unique activity patterns exhibited by many neurons with respect to the entire trajectory of the mating behavior facilitate neuronal identification when performing whole-brain imaging. Many neurons contribute to multiple sub-behaviors in different ways, leading to different correlation patterns throughout the circuit in different contexts. Functional correlations between neurons are not fixed, but explicitly depend on context and behavioral state[50]. Nevertheless, many quantitative aspects of male mating behavior may be decoded from brain-wide activity pattern.

Whole-brain imaging promises to shed light on many aspects of worm behavior, but a major hurdle is data analysis. Extracting signals with minimum motion artifact is challenging in an animal where the brain itself deforms during normal locomotion[33;35-37]. As more behaviors are studied for long periods of time (timescale of tens of minutes or even hours), data analysis needs to become increasingly automated without losing the reliability and accuracy of manual annotation (see Computational methods for understanding neural and behavioral data).

Another caveat is that, in some cases, different calcium activity patterns are encoded in different parts of the same neuron. To more easily separate traces from neighboring cells, most whole-brain imaging studies have used nuclear markers of calcium dynamics. This creates a well-separated constellation of discrete imaging volumes for all neurons, but it misses computationally relevant calcium dynamics that may occur only in the nerve fibers and processes of many neurons[51-53]. Whole-brain imaging with comprehensive nerve fiber segmentation imaging in the small worm brain is difficult to imagine with current methods. But in an animal that encodes the full range of its complex behaviors in only hundreds of neurons, the computing power of single cells should not be underestimated. The sophistication of single cells in *C. elegans* is clearly demonstrated in its motor circuit. In larger animals, networks of spinal cord neurons give rise to rhythmic and organized movements[54-57]. In *C. elegans,* single motor neuron types encode the properties of networks of cells found in larger animals[58;43]. Careful analyses of spatiotemporal properties of specific neuron classes will continue to play a vital role even with the availability of whole-brain approaches.

The fruit fly *Drosophila melanogaster*

Like *C. elegans,* the fruit fly has long been used to study the genetic basis of behavior. Since the advent of optical methods for recording brain activity using transgenic animals, the fly has been a widely used model for systems neuroscience: from its larval stage (with about 10,000 neurons) to its adult stage (with about 100,000 neurons)[59]. These two life stages have different behavioral repertoires. Larval behavior primarily consists of foraging for food and avoiding threats, while the adult fly exhibits a wider range of complex behaviors. The adult integrates visual, auditory, and chemosensory cues when flying and walking, and as it engages in social behaviors such as courtship, mating, and aggression.

Whole brain imaging in adult *Drosophila* is possible with either fast volumetric two-photon or light-field microscopy. To visualize the entire brain with cellular resolution via imaging, the fly's brain must be exposed and its head fixed with respect to a microscope, limiting its range of motion. Nevertheless, a rich set of sensorimotor behaviors can be explored with head-fixed flies in tethered flight or walking on trackballs[60].

The large size of the adult *Drosophila* brain makes it difficult to record from the whole brain at once with high spatial and temporal resolution. When whole-brain recording is performed with uniformly labeled cells, the dense packing of cell bodies and neurites makes it difficult to resolve the optical signal of individual neurons. Because it is impossible to align individual neurons across animals, comparing experiments requires computational registration of recordings from different animals to a common spatial atlas[61]. Calcium dynamics in brain-wide recordings from the adult fly are often measured from the densely packed neuropil, where each imaged voxel represents the integrated activity of many neuronal fibers. These fibers – which locally receive and transmit synaptic signals and propagate activity along their lengths – will generally have richer calcium dynamics than the cell bodies that are more distant from synaptic contacts. Imaging volumes instead of discrete neurons results in whole-brain activity measurements in the adult *Drosophila* with mesoscale resolution[62;28]. These pan-neuronal recordings in the adult fly are revealing

common principles of whole-brain function. As in *C. elegans,* large fractions of the brain show correlated patterns of activity even in the absence of stimuli[62;63].

To isolate the activity of single cells in *Drosophila,* complementary labeling approaches are often be used. Using selective drivers of gene expression, comprehensive recordings of region-level activity can be supplemented with targeted recordings from single cells and cell types of interest. Sparse labeling strategies are another option, giving the experimenter access to a subset of neurons across the brain with single-neuron resolution[64].

Brain-wide imaging in the adult fly is now being used to perform whole-brain searches for behavioral circuits that are less biased towards where sensory and motor signals should be located. A recent example is the discovery of an unexpectedly widespread brain-wide response to auditory stimuli. Components of fly courtship songs evoke activity from a diverse array of brain areas in both males and females[65]. Stimulus-evoked responses were relatively stereotyped in early mechanosensory areas of the brain, but became more variable in downstream regions. From moment to moment, different downstream brain areas respond to the same stimulus inputs. This variability is not explained by changes in the animal's instantaneous movements, suggesting that auditory information shapes, but does not alone drive, motor behavior during courtship. Internal states also affect brain-wide activity and behavior. In female flies, long-lasting internal states drive different brain activity patterns and behaviors in the presence of males: changes in receptivity to courtship as well as aggressive behaviors like shoving and chasing[66].

Brain-wide imaging is also being used to uncover mechanistic principles that likely extend to whole-brain dynamics in larger animals. A recent study of brain-wide imaging in the adult *Drosophila* brain examined the correlation between measures of metabolic activity (fluorescent indicators of intracellular molecules associated with cellular energy metabolism) and calcium activity[63]. The fMRI signal in whole-brain recording of humans and other large animals is a direct measure of changes in blood flow, which is believed to reflect changes in local neuronal activity[9]. The fact that this is empirically true in the fly suggests a general principle of brain physiology that seems to be shared by species separated by more than 400 million years of evolution.

Recently, using nuclear-localized GCaMP and oblique light sheet (SCAPE) microscopy, it has become possible to image a significant fraction of the central brain of an adult fly at single-neuron resolution as it walks on a trackball[67]. Thousands of neurons in the brain were recorded as the fly preformed a number of behaviors, including running, grooming, and flailing. These data revealed populations of neurons correlated to behavior over multiple timescales, from seconds to minutes. Different behaviors were coupled to distinct patterns of brain-wide activity, with some behaviors engaging the whole brain more strongly than others. While large fractions of the brain appeared to have activity correlated with behavior, the uncorrelated portions of the brain had high-dimensional activity. These data show that brain-wide neural activity consists of a combination of localized and broadly distributed components.

As in *C. elegans,* it is likely that when recording from neuronal nuclei alone, many signals in the neuronal processes are missed. Despite this caveat, the ability to record from thousands of neurons simultaneously in the fly brain represents a significant advance. These results also highlight a key advantage of whole-brain approaches—the ability to contextualize the activity of a single circuit within a larger network.

It is also possible to capture the activity of the whole central nervous system of an immobilized *Drosophila* larva with light sheet microscopy[68]. Whole-brain recording in a crawling *Drosophila* larva is harder because of the drastic movements and deformations of the brain in freely crawling animals[69]. The fictive motor behaviors of a brain that was surgically removed from a larva's body could be inferred from the activity of its ventral nerve cord in a recent whole-brain imaging study using light-sheet microscopy. Two-photon tracking microscopy and single-objective light microscopy have been used to follow the activity and movements of small numbers of neurons in the motor circuit of freely moving larvae[22]. As these tracking techniques gain in spatial and temporal resolution, they are likely to extend to larger circuits for behavior in the unrestrained larva.

The larval zebrafish *Danio rerio*

One vertebrate model organism shares the relatively small size, optical accessibility, and well-developed genetic toolbox of flies and worms. The larval zebrafish (*Danio rerio*) has about 100,000 neurons[70], and performs a large variety of stimulus-evoked navigational behaviors. These include hunting and prey capture, as well as threat avoidance[71]. Its brain layout has strong homologies to mammalian brains (e.g., a *bona fide* cerebellum and hypothalamus), making it a good candidate for cross-species studies[72]. Its many neurons make it difficult to identify and compare the same labeled neurons from animal to animal. Functional analysis of whole-brain imaging focuses on identifying spatial regions of the brain with coherent activity patterns aligned to a spatial brain atlas. The relatively stereotyped overall topology of the zebrafish brain facilitates alignment across individuals, allowing brain maps to be compared for different animals and different experiments with near cellular resolution[73;74].

The calcium activity of the entire brain of an immobilized larval zebrafish was first recorded with single-neuron resolution using light-sheet microscopy[75]. Even in this immobilized larva, correlated activity patterns were observed in large numbers of neurons across brain regions, and cyclic activity was observed on multiple timescales in different neurons. Since then, comprehensive recording with cellular resolution has been used to study a number of sensorimotor behaviors in immobilized and semi-immobilized animals[76]. One way to decode the motor behavior of an immobilized fish is to record the electrical activity of motor nerves in its tail during whole-brain imaging[77]. Another way is to immobilize only the head for whole-brain imaging while monitoring the free movements of the tail. Thus, a complete map of neurons and brain areas involved in various sensory to motor transformations can be obtained. Recent studies have mapped brain-wide circuits for thermosensory and optomotor responses, demonstrating the progressive computations that integrate separate sensory streams – e.g., separate images presented to the left and right eye, or the detection of warming, cooling, and absolute temperature – into purposeful motor decisions[78;79]. The

discovery of neurons that neither strongly correlate to individual sensory inputs nor motor outputs represent convergence neurons that carry out intermediate steps in information processing and non-reflexive decision-making[80].

For example, the zebrafish larva has a strong and innate optomotor response that allows it to orient when it sees a moving environment. But when zebrafish are presented with randomly moving dots with a slight bias, they accumulate and integrate motion evidence over time before deciding in what direction the dots are moving[81;82]. The zebrafish larva also performs memory-dependent behaviors including operant conditioning[83;84]. When swimming does not result in perceived movement, fish will gradually change their willingness to perform swim bouts[85]. As the larva gradually changes its decision-making, functional correlations in a distributed brain-wide network also change. These functional changes predict the outcome of decisions, and point to the distributed nature of decision-making throughout the brain[86;87;85].

Like most other animals, zebrafish larva exhibit sustained behavioral states that affect brain activity. For example, brain-wide imaging has been used in the zebrafish larva to uncover sleep signatures that resemble slow-wave sleep and rapid eye movement (REM) sleep in mammals[88]. These sleep states have the same dependence on hormone signaling as the homologous states in mammals, pointing to conserved principles in the brain-wide organization of sleep.

Behavioral states in active fish can only be discerned if the fish are allowed to exhibit behavior. One way to elicit purposeful behavior from a fish is to close the loop between perceived motor action and an applied stimulus to effectively create a virtual reality environment that can be explored by an immobilized fish. In a recent study of zebrafish larvae navigating a virtual reality environment, normal exploratory behavior was observed. However, when the system was switched to “open loop”, swim commands no longer correlated to perceived self-motion, and the fish begin swimming intensely for a period, before entering a state of futility-induced passivity[85]. Whole-brain imaging revealed the corresponding distinct brain states, and the discovery of glial cells which accumulate evidence of futility and ultimately trigger the change in behavioral state. Internal state transitions after prolonged behavioral challenges have also been demonstrated at the level of brain-wide circuits. Whole-brain imaging with prolonged behavioral challenge uncovered the progressive activation of neurons in the habenula, a brain area that controls other circuits that regulate passivity[89].

Functional whole-brain imaging studies in larval zebrafish have also enabled the discovery of neural populations with functional roles that are conserved in other vertebrates. By combining whole-brain activity with cell-type specific markers, whole-brain imaging uncovered a variety of neuromodulatory cell types that are correlated with the animal’s internal states[90]. Remarkably, homologous neuromodulatory cells in the mouse exhibited similar state-dependent dynamics as the larva, underscoring the generalization of principles learned from whole-brain imaging in small, accessible model animals.

Many complex behaviors and behavioral states only occur in unfettered animals. Certain forms of environmental feedback, such as proprioceptive or vestibular cues, cannot easily be replicated in virtual reality. One recent study of the vestibular response in an immobilized zebrafish larva was accomplished with a specialized whole-brain imaging system that rotated in its entirety[91]. Complex naturalistic behaviors, such as hunting, can only be studied in freely moving animals. The predation of *Paramecia* by zebrafish larvae is a multi-component behavior consisting of visual search, pursuit, and prey capture. Hunting requires rapid sensory processing, motor feedback, and fast context-dependent decision making to continue or abort a pursuit. High speed whole-brain imaging with microscopes that track freely-moving larvae has identified brain regions activated during prey capture[29]. Recording from freely swimming zebrafish foraging for *Paramecia* has revealed transitions between distinct brain states for exploratory locomotion versus hunting and identified the network of neurons that trigger this transition[92].

Whole-brain structural imaging

The functional imaging approaches described above provide a means of quantifying the activity of the whole brains of diverse species. The small size of the animals reviewed here is also an advantage when carrying out structural imaging: acquiring the detailed synaptic connectivity of their entire nervous systems. Determining the “wiring diagram” of the brain through structural imaging enables direct comparisons between functional activity data and neural anatomy. Connectomes thus place important constraints on the correlation structure of brain-wide neural activity. Connectomics requires serial-section electron microscopy – the only imaging modality with the throughput and resolving power necessary to reconstruct complete synaptic circuits.

C. elegans was the first animal to offer a near complete synapse-level map of its entire nervous system, a heroic feat with methodology in the 1980s[38]. An analysis of a complete circuit for behavior directly emerged from this connectome. Through systematic laser ablation and behavioral analysis, Chalfie et al. mapped the circuit for harsh touch sensitivity – a feedforward reflex that allows the worm to avoid anterior or posterior touches by rapid backward or forward movement – from sensory neurons to interneurons to motor neurons[93]. Since this early achievement, the connectome has provided an invaluable resource for mapping behavior to circuits in *C. elegans.* A larger challenge is to use the connectome to understand whole-brain activity patterns.

One approach to using whole-brain connectomes is to compare animals with connectomes with informative differences. The low throughput of whole-brain connectomics precludes doing this on a large scale for most animals. Comparative connectomics, however, has begun with the nematode *C. elegans*. The connectome has been mapped for an isogenic population of nematodes across development at different time points from birth to adulthood[39]. Substantial remodeling of synaptic circuits that is directed by a number of organizing principles and brain-wide patterns was observed. The changing connectome of the growing worm is likely to underlie changes in whole-brain dynamics and behavior that accompany its maturation. Brain-wide imaging applied to the developing worm may reveal the effect of anatomical maturation on circuit dynamics.

Connectomes of larger animals are being reconstructed. Substantial portions of the connectome of an entire *Drosophila* larval brain have been mapped, providing insights into its circuits for sensory processing, decision-making, learning and memory, and motor control[94;95]. The synapse-level connectome of an adult *Drosophila* hemibrain has recently been completed, and additional connectome maps are underway[59;96;97].

The adult *Drosophila* connectome has been used to assess brain-wide functional connectivity. The pattern of resting-state functional correlations in brain-wide calcium activity has been shown to reflect the coarse-grained structural connectivity of the fly brain (as inferred from the full anatomical wiring diagram). A similar relationship between functional and mesoscale structural connectivity has been observed in the mammalian brain, underscoring the role of synaptic connections in shaping brain-wide activity patterns across species[98].

Structural studies are underway in the brain of the larval zebrafish. Light microscopy and the integrated analysis of a large panel of sparsely-labeled transgenic fish has been used to build a comprehensive atlas of the brain with single-cell resolution[74]. Serial-section electron microscopy, albeit at lower resolution than needed for individual synapses, has been used to reconstruct the morphology of all cells and fibers in the brain[99]. With high-resolution imaging and automated analysis, complete maps of the zebrafish brain with full synaptic resolution are forthcoming[100;101].

The connectome is not sufficient to understand brain-wide dynamics. As studies of brain-wide activity repeatedly show – whether in worms, flies, or fish – the same connectome can give rise to functional correlations between neurons and across brain regions that change dramatically with environmental context and behavioral state. In *C. elegans,* we know that the wiring diagram is largely stable across isogenic individuals that exhibit the same behaviors, underscoring its functional relevance[39]. The computational properties of the brain are encoded in both its synaptic and non-synaptic pathways of communication that span multiple spatial scales – from micro-circuits to the whole nervous system – and multiple temporal scales – from seconds to animal lifetimes. Connectomes, when combined with whole-brain activity patterns at the cellular and synaptic level, will be essential for modeling brain activity.

Computational methods for understanding neural and behavioral data

Emerging methods for high-throughput connectomics, whole-brain functional imaging, and behavioral quantification are generating enormous datasets. We now have a pressing need for computational and statistical methods to aid in preprocessing, exploring, integrating, and ultimately understanding these data. Advances are being made at each stage of analysis, but much work must be done to realize the potential of modern recording technologies and the datasets they produce.

The most immediate problem is to extract biological signals of interest from the raw data. In the experimental setups described above, a common first step is to track neurons in a video of a moving animal and estimate the calcium fluorescence in each cell over time[102-104]. In

C. elegans, for example, the tracking problem is complicated by the fact that cells may come and go from the field of view, and their relative positions may change as the animal's body compresses and expands during movement. A variety of methods approach this problem with new machine learning techniques[33-37] and experimental techniques for multi-color fluorescence imaging[48]. Machine learning is also accelerating behavioral analysis and connectomics. Markerless tracking algorithms for identifying keypoints of interest on an animal's body—like the center-line of the worm, the eyes and tail of a larval zebrafish, or the legs, body, and eyes of a fruit fly—have seen considerable advances in recent years[105-109]. These methods transfer highly-tuned convolutional neural networks for human pose estimation to the animal setting with relatively little additional training. Deep learning has also been key to automatically tracing neural tissue in serial electron microscopy images for connectomics[39;59;110;96;111]. With these advances, it is now possible to measure neural and behavioral data with high resolution and to trace the neural circuits that give rise to this activity and drive motor output.

How do we gain understanding from these large-scale neural, behavioral, and connectomic datasets, once these preprocessing challenges have been surmounted? One approach is bottom-up, looking for simple, recurring patterns in the data that demand theoretical justification; the other is top-down, positing a normative theory of neural computation and hypothesizing a biological mechanism that could carry it out. These are complementary endeavors that ideally will meet in the middle[112].

Bottom-up approaches, also known as exploratory analyses[113], aid in visualizing high-dimensional data and, hopefully, discovering unexpected structure therein. Dimensionality reduction techniques, like clustering, principal components analysis, nonlinear manifold learning methods, and dynamical systems models, are common examples widely used in neuroscience[114]. Such techniques are used to identify stereotyped patterns of behavior[115;116], model their temporal dynamics[117;118], and relate neural activity to behavior[119;120]. For example, in *C. elegans* these analyses have been used to determine that immobilization alters brain-wide neural dynamics and its correlation structure[47].

Advances in machine learning continue to expand this toolkit, offering new techniques for finding low-dimensional structure in neural and behavioral data. For example, probabilistic state space models summarize high-dimensional time series data in terms of simpler latent "states" and a dynamical system that governs how states change over time[121;122]. Combined with neural networks or Gaussian processes, these approaches can find states that lie on a nonlinear manifold, or states that evolve according to nonlinear dynamics. Such methods underlie many recent techniques for modeling neural and behavioral time series[123-132]. How can we use these methods to learn about neural computation? One approach is to use nonlinear dynamical systems theory to characterize the learned dynamics in terms of linearizations around their fixed points[133]. What have these bottom-up approaches taught us? In the study of motor cortical dynamics during reaching, where many of these methods were pioneered, dynamical systems models have shown how complex single-cell tuning curves can be explained by a few population-level states[134]. In immobilized *C. elegans,* these approaches have shown how whole-brain activity can be characterized by a low-dimensional dynamical system with approximately linear dynamics corresponding to

behaviors like forward crawling, reversals, and turns[42]. As we look toward whole-brain recordings in more naturalistic behavior, state space models offer a means of relating neural and behavioral data in terms of low-dimensional, and hopefully more interpretable, latent states.

Top-down approaches, in contrast, start with a theoretical model of how a particular computation could be carried out, and from that derive predictions about neural and/or behavioral data. For example, theoretical neuroscientists hypothesized that an idealized neural circuit called a "ring attractor" could maintain an internal estimate of an animal's heading direction[135;136]. In the model, there is a population of neurons with each neuron tuned to a particular heading: its firing rate is highest when the animal is facing in its preferred direction. Through a balance of excitatory and inhibitory synapses, the population of neurons produces a "bump" of activity in the subset of similarly tuned neurons. Sensory cues and proprioceptive feedback provide external inputs to the circuit, causing the bump to move in accordance with the animal's heading. Recently, experiments have identified such a circuit in the *Drosophila* central complex[137], and remarkably the cells were physically arranged in a ring, just as the theory had predicted.

Rarely are theoretical models borne out so nicely in practice. Many of the brain's computations are too complex for closed-form, theoretical solutions. Instead, computational neuroscientists have recently turned to "task-based" modeling, which leverages artificial intelligence and deep learning[138-141]. The idea is to model an artificial agent performing the same computation (i.e. task) as the animal, but using an artificial neural network in place of a biological one. Rather than solving for the optimal artificial network weights analytically, task-based modeling uses stochastic gradient descent to search for an approximately optimal configuration. The trained artificial agent offers a reference point for studies of biological nervous systems. In particular, the "neural activity" of the artificial agent (i.e. the activation of units in its artificial neural network) offers a prediction of neural activity in the biological organism. The key idea is that it is often easier to identify the computational problem and the architectural constraints than it is to solve for the theoretically optimal solution, and deep learning algorithms can solve the hard problem of finding an optimal network weights for a given task. In this sense, task-based modeling offers a new approach to connecting top-down theories of computation to complex neural, behavioral, and connectomics data, opening exciting new frontiers for computational and theoretical neuroscience.

Discussion

Whole-brain imaging is now an important tool in systems neuroscience. Common themes are emerging in studies of different animals across different behaviors, pointing to shared principles in the brain-wide representation of behavior[142]. Whole-brain imaging may be necessary to understand the systems-level organization of natural behaviors as, whenever it has been applied, representations of behavior have proved to be more widely distributed that one might naively expect. Neurons and neuronal circuits are engaged in different ways during different behaviors. Understanding the functional correlations across circuits requires explicit integration of whole-brain imaging with quantitative descriptions of behavioral dynamics. Whole-brain imaging also needs to be coupled with fine-grained analyses of

individual neurons and their connectivity to capture and interpret computational dynamics at all relevant spatial scales.

This problem of understanding brains and behavior is naturally exciting for physicists. The technical demands of experiments and the challenges of understanding large and complex datasets have progressed to the point that collaboration between experimentalists and theorists in neuroscience and biophysics is needed in many of these whole-brain studies. The same relationship between theory and experiment that characterizes many areas of physics will advance the field of whole-brain imaging. Theorists are now making useful and interesting predictions, and experimentalists can test them by leveraging the growing toolbox of molecular, cellular, and structural perturbations available in genetically accessible model organisms. Here, we describe areas where experimental and theoretical physicists can help to move the field forward, either with technological advances or mathematical modeling.

Functional whole-brain recording methods: outlook and challenges

The advancement of microscopy techniques such as two-photon, confocal, structured illumination, and light-sheet have enabled functional imaging of the entire brains of *C. elegans, Drosophila,* and larval zebrafish. Each technique offers a different ratio of the speed-resolution trade-off. Combining techniques such as spinning-disk confocal microscopy and light-field microscopy[30], two-photon with light-sheet microscopy[143], or incorporating deep-learning techniques for resolution enhancement[31] can partially alleviate the speed-resolution trade-off. While the small size of *C. elegans* enables functional whole-brain imaging in freely moving animals at high speeds and at single-cell resolution[46;33], and recent work in *Drosophila* has enabled high-speed recording of flies walking on a ball with single-cell resolution[67], the development of microscopy systems capable of recording functional whole-brain datasets with cellular resolution at speeds that match the multiple timescales of neural and behavioral dynamics remains a challenge in larger organisms.

Another challenge for whole-brain imaging in freely moving animals is the improvement of tracking algorithms. *C. elegans* and zebrafish display movements of high complexity[144] and tracking has largely relied on proportional error-correction control software[46;33;145;29]. This method compensates for changes in position but does not compensate for the deformation and changes in brain orientation. In the case of *C. elegans,* the brain deforms as the worm moves, making it difficult to track the identity of the neurons being recorded over time. Recent studies, train deep neural networks to recognize the configurations the brain adopts in different worm postures. This approach enables tracking neurons with ~ 74% accuracy[146;147]. In larval zebrafish, the brain does not undergo significant deformation during free swimming behavior and data analysis relies on mapping the recorded brains onto a reference brain atlas that enables near single cell resolution alignment[73;145]. Nonetheless, tracking the identity of neurons in different fish remains an unresolved challenge.

Body posture dynamics in *Drosophila* involves the use of six legs and a pair of wings, making posture dynamics segmentation a complex computational challenge. In recent years, the development deep neural network techniques for pose estimation[148;106;149], and unsupervised techniques for body position dynamics[115] has enabled the development of predictive models of behavior with higher spatial and temporal resolution[144]. Incorporating

these new developments in animal pose estimation and predictive models of behavior[150] into tracking control algorithms will significantly improve the throughput and quality of whole-brain datasets in behaving flies.

Physics-based theoretical frameworks to merge levels of neural computation

Understanding the way in which high-level computational features of brain processing such as decision-making algorithms, sensorimotor transformations, and internal state trajectories emerge from the low-level activity or molecular properties of individual neurons requires the development of theoretical and computational tools which span top-down and bottom-up modeling approaches.

Physics has long navigated different levels of abstraction of natural phenomena. In non-living matter, theoretical approaches have established satisfactory descriptions of behavior from the level of subatomic particles to that of entire galaxies. In living matter, physics has also succeeded in bridging different levels through coarse-graining. For example, in the study of bacterial chemotaxis[151], models that describe how operon structure determines gene expression have been incorporated into higher-level models that describe the behavior of populations of freely swimming bacteria[152]. This multiscale theoretical approach merges physics-based models of molecular networks with physics-based models of random walks. It led to understanding the way in which correlation structure in gene expression can shape the distribution of behaviors in a bacterial population, and the manner in which this determines environmental fitness[153].

In neuroscience, physics-based models exist at many scales, from descriptions of ion channels and detailed Hodgkin and Huxley models of neurons and small circuits[154;111] to maximum entropy models of whole-brain activity[155] and phenomenological models of decision-making and behavior[144]. Theoretical efforts to understand higher-level brain function from whole-brain activity and connectomics should not be limited to dynamical systems that transform neural dynamics into behavioral dynamics. They should also incorporate levels of abstraction where the contribution of circuit properties at multiple scales – such as network motifs, control algorithms, relative timescale constraints, and weak linkage – can be tested. This challenge could be tackled, for example, by starting with computational multiscale agent-based models that incorporate different scales of abstraction and then moving to analytical models that capture relevant phenomena in the range of scales and parameters that are relevant to a specific scientific question.

Conclusion

Whole-brain imaging was made possible by technological advances in optics, genetics, fluorescent sensors, and computational image analysis. These whole-brain datasets have allowed novel theoretical frameworks to be compared against measured data. Looking forward, we hope that continued advancements in both experimental and theoretical methods will enhance our understanding of brain-wide computation.

The field of whole-brain studies of animal behavior has been initiated with animals – invertebrates and vertebrates – that evolutionarily diverged over 400 million years ago. The

complex behaviors exhibited by worms, flies, and fish are analogous to behaviors studied in larger animals. In these larger animals, however, it is only possible to study these behaviors with more compartmentalized approaches. The identification of common principles in brain dynamics and behavior in these genetically tractable small model organisms are likely to represent principles that are widely shared across the animal kingdom.

Neuroscience has historically been constrained by the available technologies to reductionist approaches to understanding behavior, recording from small numbers of neurons in controlled settings. Ethology, on the other hand, has relied on careful observations to study natural animal behavior. Determining the neural basis of animal behavior has been a long-standing interest of both fields. Since behavior often engages widely distributed brain circuits, however, until recently it has not been possible to simultaneously capture behavior and high-dimensional neural activity[150]. Advances in physics, biotechnology, and computer science have allowed this gap to be bridged. Whole-brain approaches to brain dynamics and structure are now opening a new and interdisciplinary field: studying the neural basis of natural behavior.

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Box 1:

Model organisms for whole-brain imaging during natural behavior

	Nematode worm *C. elegans*	Zebrafish *Danio rerio*	Fruit fly *Drosophila melanogaster*
			
Number of neurons	300	10^5 (larva)	10^4 (larva), 10^5 (adult)
Behaviors studied	crawling escape response mating	swimming light taxis prey capture	walking flight mating auditory responses
Experimental access	single-neuron resolution identifiable neurons	single-neuron resolution aligned brain atlas	brain region resolution aligned brain atlas

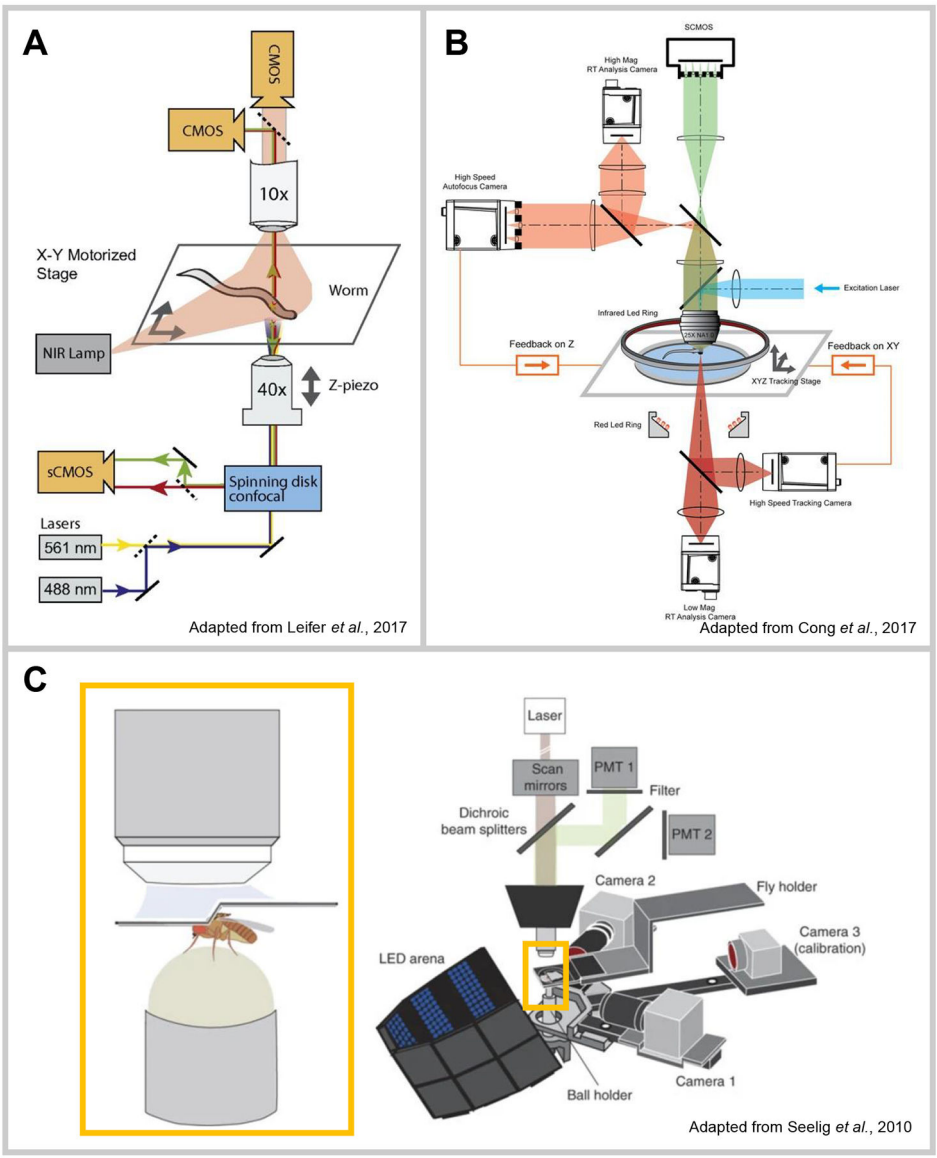

Figure 1: Recording from the brains of behaving animals.

(A) A *C. elegans* worm crawls freely on a motorized stage. A low-magnification 10x objective captures the animal's entire body to record posture and behavior, while a high magnification 40x objective records calcium activity from the animal's brain. Real-time feedback keeps the animal in the objectives' field of view. Adapted from Nguyen et al., 2016. **(B)** A larval zebrafish swims in a thin, water-filled nchamber. A high-speed, low-magnification optical setup tracks the animal's motion, while a high-resolution light-field setup records whole-brain calcium activity.Real-time feedback in all three dimensions keeps the animal's brain in the field of view. Adapted from Cong et al., 2017. **(C)** An adult fruit fly is tethered and placed on an air-cushioned ball. A high-resolution objective allows for two-photon excitation and recording of calcium activity from the animal's head. The fly is free to walk in any direction on the ball, with low resolution cameras recording the animal's

posture and behavior. Visual and auditory stimuli are presented to the animal while it is on the ball. Adapted from Seelig et al., 2010.

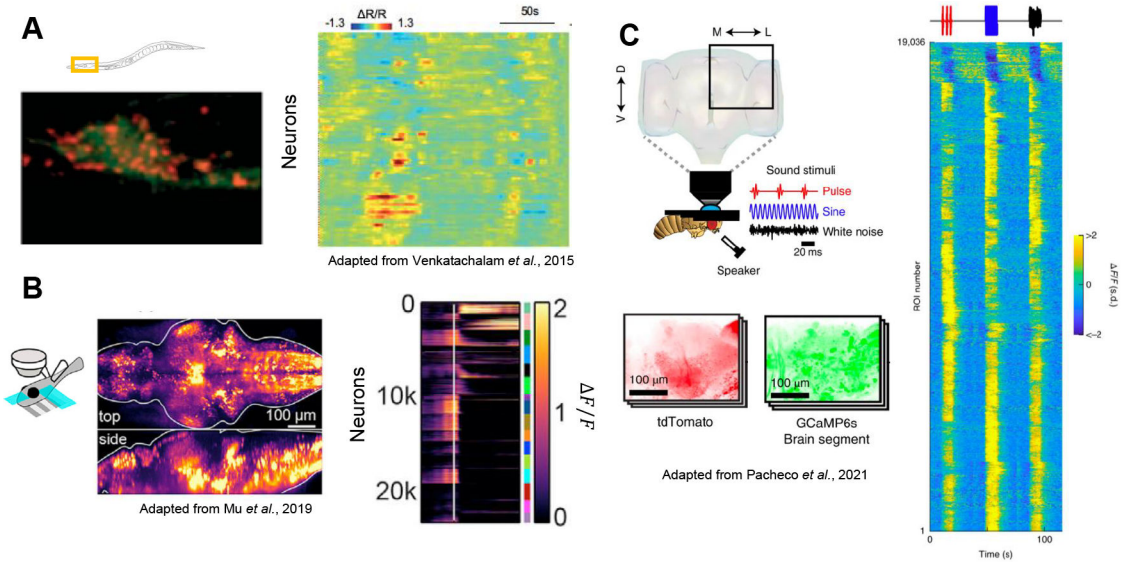

Figure 2: Samples of pan-neuronal recordings in behaving animals.

(A) A sample image of the brain of *C. elegans* (left), labeled with pan-neuronal cytosolic GCaMP6s and nuclear-localized Tag-RFP. Normalized activity traces (right) of 84 neurons in a freely crawling worm. Adapted from Venkatachalam et al., 2015. **(B)** Top and side views of the brain of a larval zebrafish (left), labeled with GCaMP6f. Activity of segmented neurons in the brain (right) during fictive swim behavior. Adapted from Mu et al., 2019. **(C)** On the left, a schematic of volumetric imaging of the brain of an adult *Drosophila* being presented with auditory stimuli. The brain is labeled with GCaMP6s and tdTomato. To the right are responses from recorded regions of interest (ROIs) to auditory stimuli. Adapted from Pacheco et al., 2021. *AL note: we may want to reach out to the authors so we can plot higher-resolution neuron trace plots in a consistent color scheme*

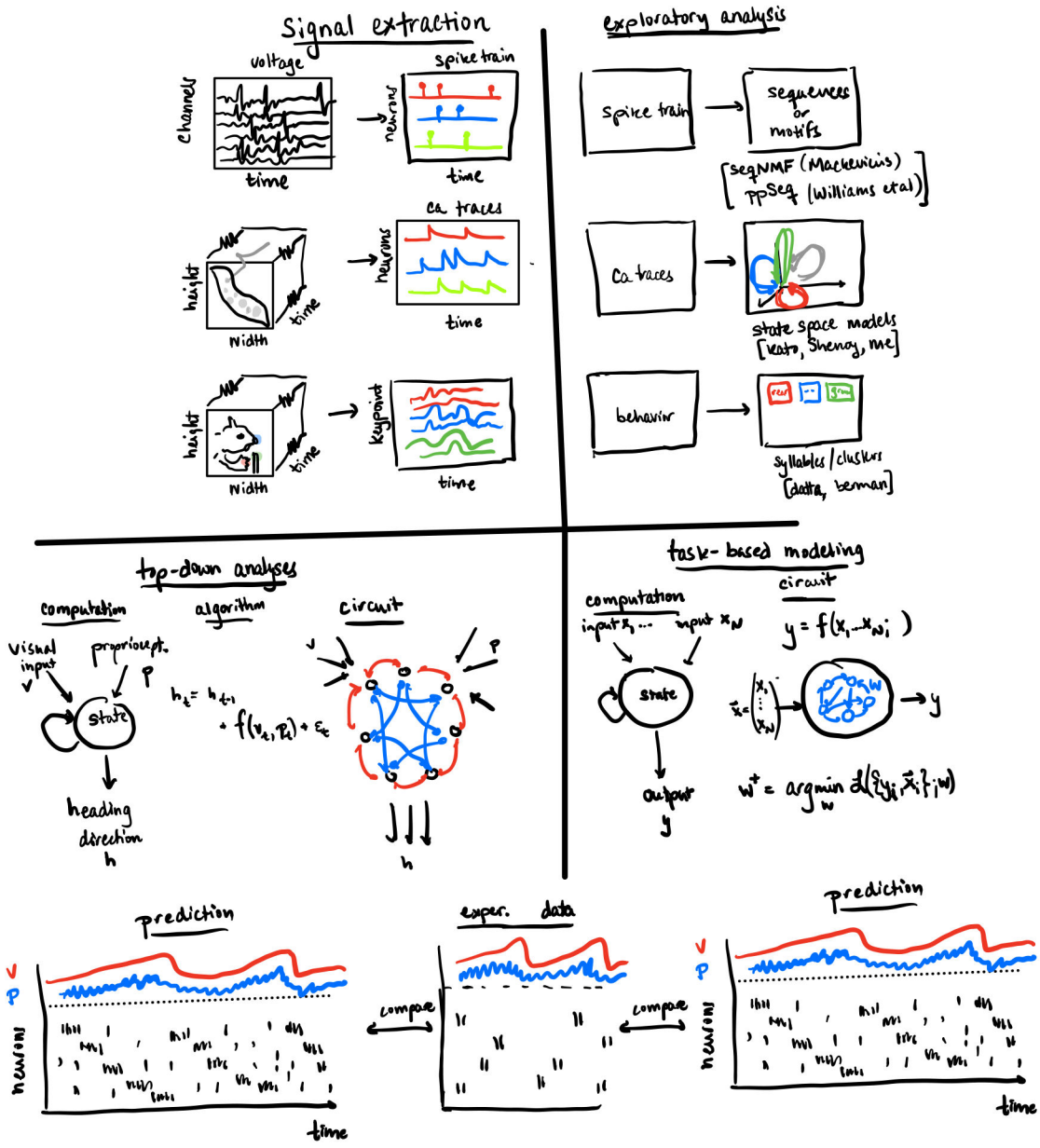

Figure 3: Computational methods for neural and behavioral analysis.

Top Left: The first challenge is to develop statistical methods to extract biological signals of interest from raw data. For example, extracting the times of action potentials (i.e. “spikes”) from extracelluar voltage recordings, demixing and deconvolving calcium fluorescence traces, or tracking body parts in videos. *Top Right:* Computational models for exploratory analysis aim to reveal simplifying structure in high dimensional signals, like repeated sequences of spikes, low dimensional trajectories of neural activity, or clusters of stereotyped behaviors. *Bottom left:* Top-down analyses hypothesize an algorithm and circuit implementation to solve a computational problem, like tracking heading given visual inputs and proprioceptive feedback. The model makes predictions about neural activity that can be tested against measured data. *Bottom right:* Rather than hand-tuning an algorithm and

circuit, task-based modeling learns a circuit to solve a particular computation by minimizing a loss function. This relatively new approach offers an indirect way of making testable predictions of neural activity.