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Review

Bioelectronics for Millimeter-Sized Model Organisms

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Advances in microfabrication technologies and biomaterials have enabled a growing class of electronic devices that can stimulate and record bioelectronic signals. Many of these devices have been developed for humans or vertebrate animals, where miniaturization allows for implantation within the body. There are, however, another class of bioelectronic interfaces that exploit microfabrication and nanoelectronics to record signals from tiny, millimeter-sized organisms. In these cases, rather than implanting a device inside an animal, animals themselves are loaded in large numbers into bioelectronic devices for neural circuit and behavioral interrogation. These scalable interfaces provide platforms to develop new therapeutics as well as better understand basic principles of bioelectronic communication, neuroscience, and behavior. Here we review recent progress in these bioelectronic technologies and describe how they can complement on-chip optical, mechanical, and chemical interrogation methods to achieve high-throughput, multimodal studies of millimeter-sized small animals.

ADVANTAGES OF MILLIMETER-SIZED ORGANISMS

Small model organisms such as *Caenorhabditis elegans*, *Drosophila*, and zebrafish have been a cornerstone for understanding principles of neurobiology, behavior, genetics, and development (Davis, 2004; Sattelle and Buckingham, 2006; Stewart et al., 2014). Despite their small nervous systems and less anatomical complexity compared with that of mammalian models, there are significant advantages in using millimeter-sized animals to answer fundamental questions in neurobiology (Figure 1).

Microscopic animals exhibit a number of basic behaviors including sleep, taxis, mating, feeding, and basic learning (Figure 1). Using these behaviors, researchers can interrogate fundamental properties of neural circuits such as multisensory integration and sensorimotor transformations (Clark et al., 2013; Ghosh et al., 2017; Kaplan et al., 2018). Among the many behaviors in these animals that enable neural circuit dissection are contractions in the cnidarian *Hydra* (Dupre and Yuste, 2017; Passano and McCullough, 1964; Tzouanas et al., 2019); thermotaxis, behavioral state transitions, and sleep in *C. elegans* (Butler et al., 2014; Cho and Sternberg, 2014; Clark et al., 2006; Flavell et al., 2013; Gallagher et al., 2013; Garrity et al., 2010; Hawk et al., 2018; Hedgecock and Russell, 1975; Hill et al., 2014; Kaplan et al., 2019; Li et al., 2014; Raizen et al., 2008); circadian rhythms and social behaviors in *Drosophila* (Anderson, 2016; Artiushin and Sehgal, 2017; Guo et al., 2018; Kayser and Biron, 2016; Lim et al., 2014; Saigusa et al., 2002; Stockinger et al., 2005); and sensorimotor integration and sleep in zebrafish (Ahrens et al., 2012; Chen et al., 2018; Cong et al., 2017; Gandhi et al., 2015; Haesemeyer et al., 2018; Naumann et al., 2016; Oikonomou and Prober, 2017; Severi et al., 2014; Vladimirov et al., 2018; Zhdanova et al., 2001; Zimmerman et al., 2008). Furthermore, many of these behaviors show a surprising resemblance to mammalian behaviors at the molecular level (Gandhi et al., 2015; Monsalve et al., 2011; Siebert et al., 2019; Singh et al., 2014; Trojanowski and Raizen, 2016; Van Buskirk and Sternberg, 2007; Zimmerman et al., 2008), demonstrating that although these animals are anatomically diverse, there are many molecular and genetic factors that are conserved across phylogeny.

Another significant advantage of millimeter-sized animals is the percent coverage of the nervous system that can be simultaneously monitored using genetically encoded calcium indicators (Figure 1) (Broussard et al., 2014; Chen et al., 2013). In mammalian neuroscience, a significant neurotechnological goal is to increase the number of neurons that can be simultaneously recorded in hopes of revealing basic principles of large networks of neural circuits (Insel et al., 2013). State-of-the-art techniques for recording neural activity in rodent models with Neuropixels probes have achieved hundreds of single units simultaneously and up to 30,000 units over multiple experiments (Allen et al., 2019; Juavinett et al., 2019; Jun et al., 2017; Steinmetz et al., 2019), whereas more than 10,000 neurons can be simultaneously accessed using two-photon calcium imaging with the tradeoff of significantly lower temporal resolution (Pachitariu et al., 2016; Stringer et al., 2019a, 2019b, 2019c). However, due to the large size of the rodent brain, even these impressive technologies

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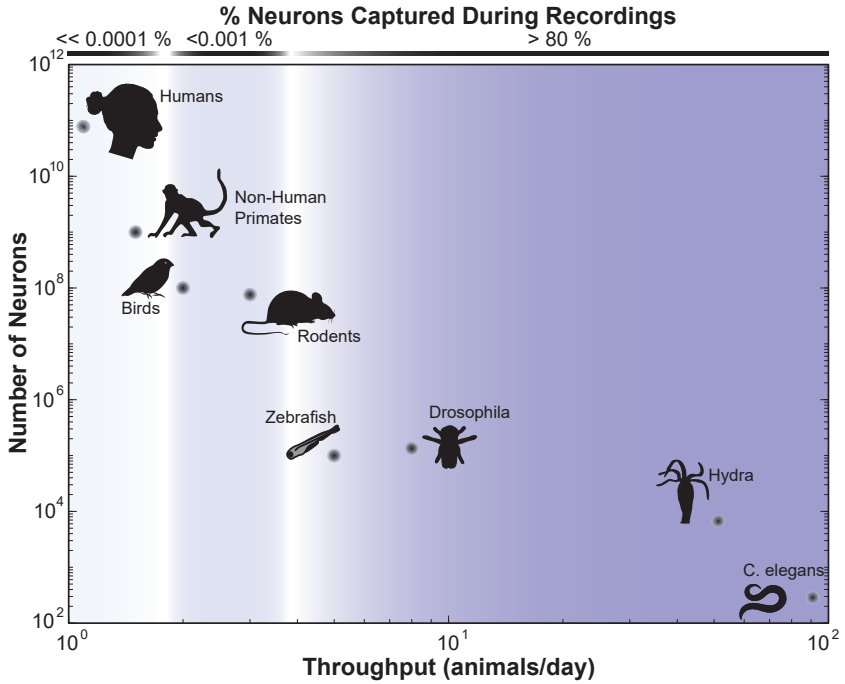

Figure 1. Tradeoffs of Small-Model Organisms for Neurobiology

Common model organisms in neurobiology and estimated tradeoffs between experimental throughput and the number of neurons in the nervous system. On the top axes, we also denote the approximate percentage of single neurons that can be simultaneously measured during experiments. Although the structure of the nervous system in millimeter-sized animals is significantly different than the mammalian brain, many molecular and cellular properties are conserved, and these animals show orders of magnitude improvements in experimental throughput and the number of neurons that can be simultaneously recorded.

record from less than 0.001% of the neurons in the rodent brain (Williams, 2000). To better understand information processing in an entire nervous system at cellular resolution, scientists can turn to millimeter-sized animals. The compact size and optical transparency of small organisms such as *C. elegans*, zebrafish larvae, drosophila larvae, and *Hydra* enable whole-brain—and even whole-nervous-system—imaging (Ahrens et al., 2013; Bouchard et al., 2015; Dupre and Yuste, 2017; Kato et al., 2015; Keller et al., 2014; Nguyen et al., 2015; Prevedel et al., 2014; Venkatachalam et al., 2015; Voleti et al., 2019). Whole-brain imaging systems for *C. elegans* regularly achieve cellular resolution, recording the activity of 60–130 neurons in the worm head simultaneously, thus capturing the activity of 20%–40% of the nervous system (Kato et al., 2015; Nguyen et al., 2015; Nichols et al., 2017; Venkatachalam et al., 2015). Light-sheet and two-photon microscopy in zebrafish also achieve cellular resolution during calcium imaging and can capture up to 80% of neurons in a single animal (Ahrens et al., 2013; Haesemeyer et al., 2018; Keller et al., 2014; Vladimirov et al., 2018). Finally, whole-nervous-system imaging in *C. elegans* and *Hydra* has been employed using light-field microscopy, SCAPE imaging (swept, confocally aligned planar excitation), and wide-field epifluorescence imaging, recording the activity of nearly every neuron in the nervous system simultaneously (Dupre and Yuste, 2017; Prevedel et al., 2014; Voleti et al., 2019). These powerful techniques can be combined with both behavioral and computational analyses to reveal the fundamental features of neural circuit activity and understand how the ensemble activity of the brain results in behavior (Ahrens et al., 2012; Chen et al., 2018; Cong et al., 2017; Haesemeyer et al., 2018; Kaplan et al., 2019, 2018; Nguyen et al., 2015; Scholz et al., 2018; Severi et al., 2014; Vladimirov et al., 2018; Zaslaver et al., 2015).

Finally, a powerful advantage of millimeter-sized animals is the experimental throughput that can be achieved with scalable microfabricated devices and semi-automated experimentation (Figure 1). Because of their small size, experimental approaches can be developed to record behavior, electrophysiology, or neuronal calcium activity from dozens of organisms in parallel (Gupta and Rezai, 2016; San-Miguel and Lu, 2013; Yanik et al., 2011). In fact, experimental throughput can often be scaled to hundreds of animals per day, orders of magnitude more than is typically achieved with rodent models. For example,

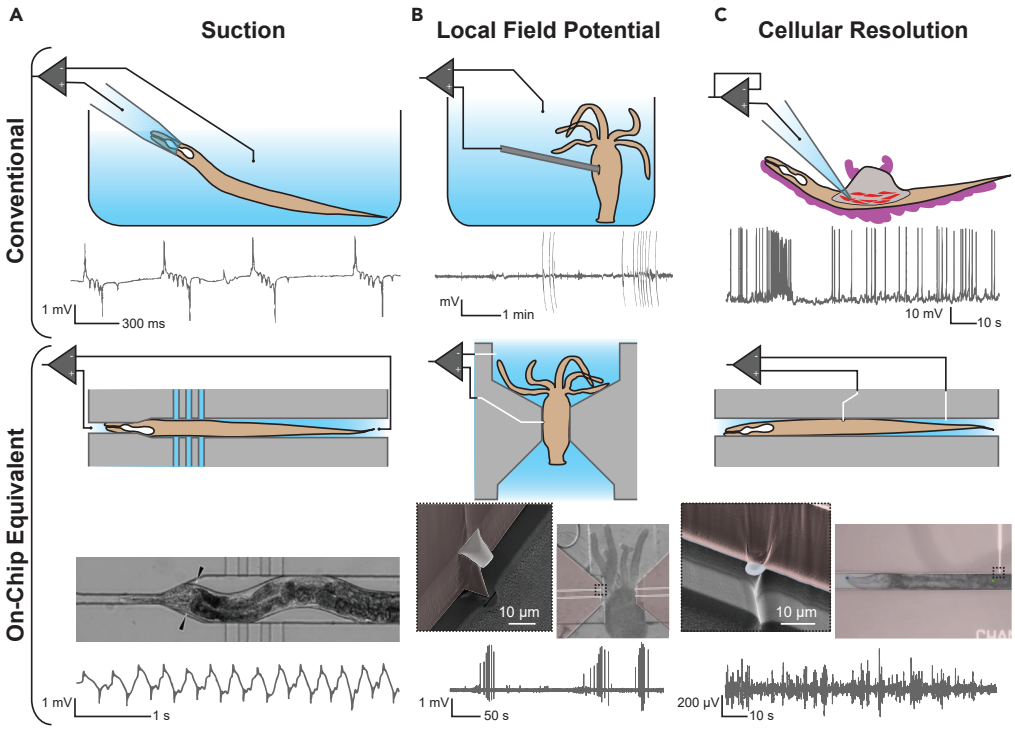

Figure 2. Examples of Bioelectronic Interfaces

Top row depicts conventional technologies for recording from the nervous system of *Hydra* and *C. elegans*. Bottom row shows these same recording modalities but adapted for on-chip recordings in fluidic microdevices that significantly increase experiment throughput.

(A) Suction electrodes for recording neuromuscular activity from the *C. elegans* pharynx.

(B) Electrodes pressed into the *Hydra* body can record local field potentials and contraction bursts.

(C) Nano-SPEAR electrodes tightly pressed into the *C. elegans* body wall record similar spiking activity as patch-clamp electrophysiology.

Panel A (top) adapted from Lockery et al. (2012) with permission from the Royal Society of Chemistry and (bottom) adapted from Raizen and Avery (1994). Panel B (top) adapted from Passano and McCullough (1964) with permission from The Company of Biologists and (bottom) adapted from Badhiwala et al. (2018). Panel C (top) adapted from Gao and Zhen (2011) and (bottom) adapted from Gonzales et al. (2017).

microfabricated devices with throughputs of at least 20–1000 animals/day for *C. elegans* have been developed that monitor behavior (Albrecht and Bargmann, 2011; Churgin et al., 2017; Hulme et al., 2010; Swierczek et al., 2011), record single-neuron calcium activity (Chokshi et al., 2010; Chronis et al., 2007; Larsch et al., 2013), detect synaptic morphological changes (Chung et al., 2008; Crane et al., 2012), record electrophysiological activity (Gonzales et al., 2017; Hu et al., 2013; Lockery et al., 2012), and screen for drug efficacy and side effects (Cornaglia et al., 2016; Mondal et al., 2016). Likewise, similar technologies fabricated at the microscale have been developed for *Hydra* (Badhiwala et al., 2018; Dexter et al., 2015), drosophila larvae (Bernstein et al., 2004; Chung et al., 2011; Levario et al., 2013; Lucchetta et al., 2006), and zebrafish (Chang et al., 2012; Pardo-Martin et al., 2013, 2010) for monitoring the behavior of animals with a throughput reaching tens of animals/days.

These three main advantages—conserved fundamental behaviors, monitoring of large-scale brain activity, and scalable experimental paradigms—make small, millimeter-sized animals powerful models for understanding the bioelectronic signaling of the nervous system. However, an under-explored area of technology development is using advanced materials and nanoelectronic interfaces to record bioelectric activity from small organisms. Conventionally, bioelectronic devices are designed for implantation within animals to stimulate neurons or muscles or record the millivolt-level signals they produce. In the case of millimeter-sized organisms we can use similar technologies to enable multimodal devices that house small organisms for reading and writing electrophysiological activity at a large scale (Figures 2 and 3). These advanced technologies, when coupled with the advantages of small model organisms, empower researchers with the

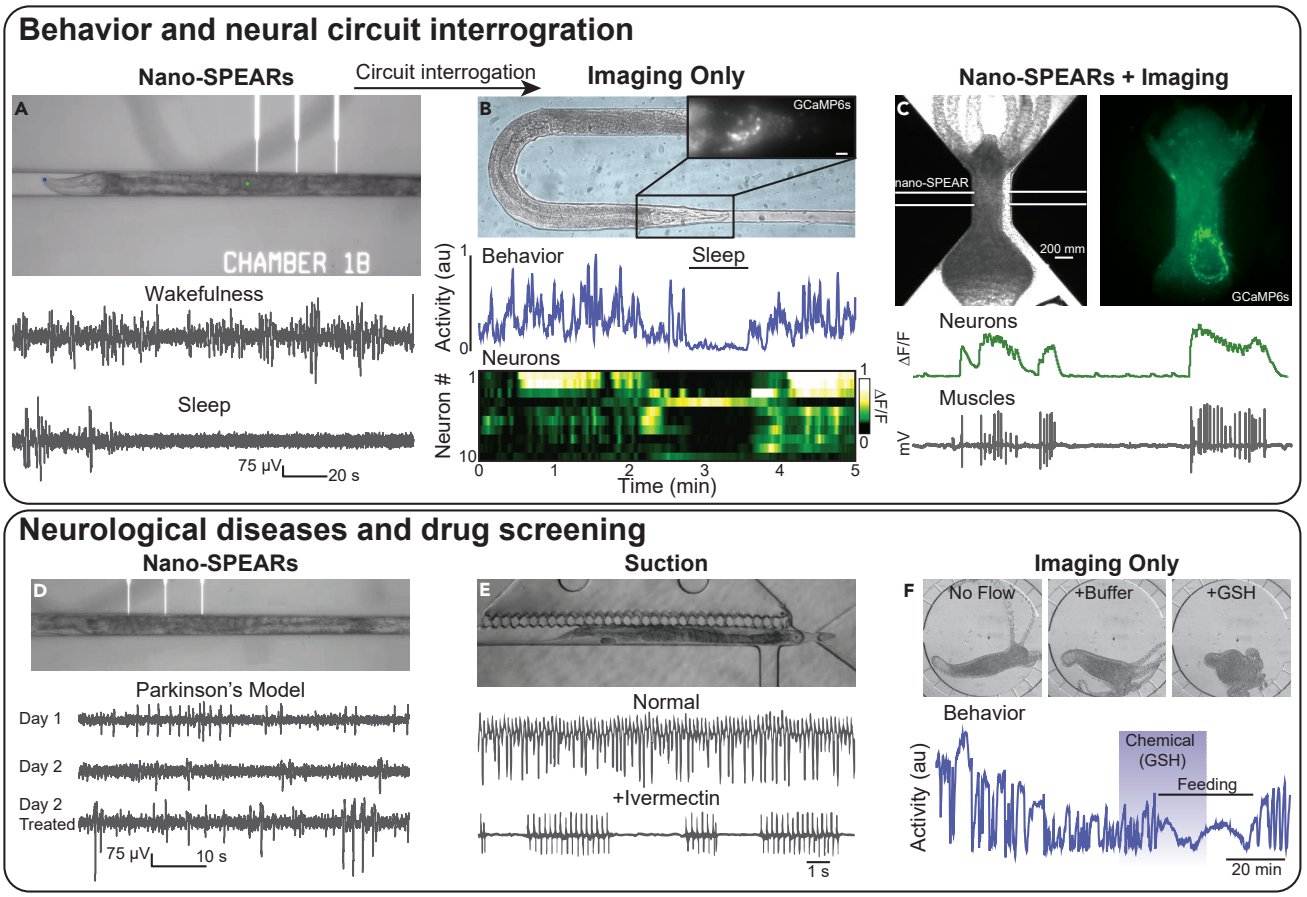

Figure 3. Bioelectronics for Studying Neural Circuits, Behavior, Disease Models, and the Effects of Drug Candidates

(A) Adult *C. elegans* partially immobilized in a microfluidic chamber for muscle cell recordings with nano-SPEARs. These recordings led to the discovery of a behavioral state transition between sleep and wakefulness.

(B) Bioelectronic recordings in (A) are complementary to imaging methods for behavioral monitoring and calcium imaging. These techniques were used to further dissect the neural mechanisms underlying microfluidic-induced sleep. (Top) Adult *C. elegans* partially immobilized in a microfluidic chamber during whole-brain GCaMP6s imaging (inset scale bar is 5 μm). (Bottom) The partial immobilization enables simultaneously monitoring animal behavior and neural activity during spontaneous sleep-wake state transitions.

(C) *Hydra* partially immobilized in a hybrid bioelectric-fluidic device for simultaneous behavior, electrical recordings, and whole-animal calcium imaging of neural activity. Contraction bursts correspond to dramatic increases in calcium activity in specific groups of neuronal cells. These bursts in fluorescence correspond to simultaneously recorded bursts of electrical activity in the muscle cells. Electrical measurements can be captured with high temporal resolution using nano-SPEARs.

(D) Nano-SPEAR muscle cell recordings in a *C. elegans* model for Parkinson disease. Protein aggregation deteriorates spiking activity as adults progress from day 1 to day 2; however, this phenotype can partially be rescued by a clioquinol drug treatment.

(E) *C. elegans* partially immobilized in a microfluidic device for pharyngeal recordings during drug stimulation. Ivermectin inhibits muscle and neural activity by activating glutamate-gated chloride channels, therefore inhibiting pharyngeal pumping and dramatically changing the waveform shape and frequency.

(F) Similar to (A) and (B), on-chip studies are not limited to bioelectronic recordings. Here, microfluidic immobilization of *Hydra* enables chemical stimulation without mechanically perturbing the animal while simultaneously monitoring behavior. Reduced glutathione (GSH) induces the feeding response, which leads to inhibition of contractile movements.

Figures adapted from: A (Gonzales et al., 2017), B (Gonzales et al., 2019), C (Badhiwala et al., 2018), D (Gonzales et al., 2017), E (Hu et al., 2013; Ménez et al., 2019), F (Badhiwala et al., 2018).

tools to better understand the link between neural activity and behavior and develop high-throughput screening technologies for therapeutics.

TYPES OF BIOELECTRONIC INTERFACES TO SMALL ANIMALS

Bioelectric interfaces to millimeter-scale animals exist largely in three experimental regimes for recording local-field potentials or cellular-resolution activity (Figure 2A): electrodes that create high seal resistances

through suction (Hu et al., 2013; Lockery et al., 2012), three-dimensional (3D) electrodes that are pressed (Gonzales et al., 2017) or driven (Eimon et al., 2018; Vitale et al., 2018) into the animal body, and two-dimensional (2D) electrode arrays placed externally near the animal's nervous system as they produce bioelectric signals (Cho et al., 2017a; Meyer et al., 2016; Yu et al., 2012).

Micropipette suction electrodes were some of the first devices developed for small animals and initially targeted the neuromuscular activity driving the *C. elegans* pharynx, a feeding organ that ingests food through coordinated muscle pumping (Avery and You, 2012; Raizen and Avery, 1994). In this interface, animals are flushed into microfluidic chambers and trapped in separate modules (Figure 2A) (Hu et al., 2013; Lockery et al., 2012). Using tiny microchannels, a tight seal can be formed around the worm head and a differential amplifier used to record the ensemble activity of neurons and muscles, which drives stereotyped waveforms during pharyngeal pumping (Hu et al., 2013; Lockery et al., 2012; Ménez et al., 2019; Russell et al., 2019; Sanders et al., 2017).

Two-dimensional microelectrode arrays (MEAs), much as those used to record from cultured cells (Stett et al., 2003), can also be tailored to record bioelectric signals from behaving animals. For example, patterned MEAs can interface with microfluidic channels tailored to trap animals such as zebrafish larvae (Hong et al., 2016). These interfaces record relatively non-specific local-field potentials such as EEG signals in zebrafish (Cho et al., 2017a; Meyer et al., 2016; Yu et al., 2012) but do so scalably and non-invasively.

Finally, 3D nanoelectrodes, termed "nano-SPEARs" (nanoscale, suspended electrode arrays), have been developed for interfacing with microscopic animals for bioelectric recordings at a more local scale compared with suction or 2D MEA configurations (Gonzales et al., 2017). In these hybrid nanoelectronic/fluidic devices, nano-SPEARs horizontally protrude from the walls of microfluidic chambers (Figures 2B and 2C). Animals are pressed and immobilized against these nanoprobes, enabling electrophysiological recordings of muscle activity in *C. elegans* and *Hydra*. Importantly, these electrodes can be fabricated with sizes significantly smaller than the diameter of a single cell and tightly press against the outer walls of animals (Figures 2B and 2C). This configuration takes advantage of the high-aspect ratio of nano-SPEARs, much like previously reported nanoelectrodes developed for recording activity from single neurons and cardiomyocytes (Abbott et al., 2019, 2017; Duan et al., 2012; Lin et al., 2014; Robinson et al., 2012; Tian et al., 2010; Xie et al., 2012), and enables recording *in vivo* electrophysiological activity from only a few muscle cells in *C. elegans* (Gonzales et al., 2017), but can also record local field potentials in *Hydra* (Badhiwala et al., 2018).

These bioelectronic interfaces provide many notable advantages over conventional technologies and other recording modalities. For example, on-chip platforms are not only scalable but can also be automated for significantly higher throughput experiments compared with manual techniques. Furthermore, new technologies such as nano-SPEARs are also less invasive than conventional methods and do not require animal dissections. Finally, when compared with optical measurement of neural and muscle activity, such as calcium imaging, electrical techniques have the significant advantages of being compatible with non-transparent animals and do not require genetic or chemical manipulation. Therefore, advances in millimeter-scale bioelectronics have the potential to generate automated, high-throughput experiments in both conventional laboratory animals and non-model organisms.

BIOELECTRONIC APPLICATIONS

Suction, 2D, and 3D electrode configurations provide a powerful set of tools to study the electrogenic signaling in millimeter-sized animals with a range of applications in the basic and applied sciences. For example, these platforms can be used to probe fundamental electrophysiology, the neural circuits driving behavior, as well as study neurological diseases, search for drug targets, and screen for drug treatments. In this section, we highlight the role of bioelectronics in each of these applications. In this discussion we describe how more comprehensive experiments are possible by combining bioelectronics with existing on-chip recording modalities, such as calcium imaging and behavioral monitoring.

As discussed in the "Advantages of Millimeter-Sized Animals" section, millimeter-sized animals exhibit many conserved behaviors such as locomotion, feeding, sleep, and taxis. Due to their simple nervous system these animals are excellent models to study the neural circuit basis of behavior, particularly from the viewpoint of whole-brain recordings (Figure 1). An excellent example is whole-brain calcium-imaging recordings

from *C. elegans* confined in microfluidic devices (Kato et al., 2015; Nichols et al., 2017; Skora et al., 2018). Notably, the worm connectome is fully mapped and stereotyped across animals (Cook et al., 2019; White et al., 1986). By cross-referencing neuron locations in the imaging data with the connectome, researchers can know the identity of a fraction of the individual neurons in their data (Kato et al., 2015; Nichols et al., 2017; Scholz et al., 2018), and continuing advances in transgenics and fluorescent indicators may unlock the identifies of nearly every recorded neuron (Yemini et al., 2019). The combination of *C. elegans* whole-brain imaging, behavior, and electrophysiology can provide a versatile platform for studying the neural circuit basis of behavior. For example, nano-SPEAR electrophysiological recordings in adult worms led to the discovery of a microfluidic-induced sleep state (Gonzales et al., 2017) (Figure 3A), which was further dissected with behavioral assays, genetic manipulations, and whole-brain imaging (Figure 3B) (Gonzales et al., 2019). In addition, these recording modalities can be combined with the precise environmental control offered by small microdevices, giving the ability to study how nematode circuits integrate external environmental cues and drive behavioral-state transitions, such as sleep-wake switching (Figure 3B) (Gonzales et al., 2019; Nichols et al., 2017; Skora et al., 2018).

Bioelectronic platforms combined with calcium imaging can also be applied to other transparent, millimeter-sized animals such as *Hydra* (Figure 3C) (Badhiwala et al., 2018; Tzouanas et al., 2019). As is the case for *C. elegans*, studies of *Hydra* may help researchers to understand how neural circuits distributed throughout the nervous system regulate simple behaviors such as locomotion and feeding (Figure 3C). However, the unique anatomy of *Hydra* compared with animals with bilateral symmetry such as *C. elegans* and zebrafish offer opportunities to study neural circuits in animals with very different neural architectures and body plans. Unlike the nervous systems of *C. elegans*, *Drosophila*, and zebrafish that are relatively static and stereotyped, the *Hydra* nervous system is highly regenerative, variable between animals, and can dynamically change throughout the lifetime of an individual animal (Bode et al., 1973; Gierer et al., 1972; Tzouanas et al., 2019). This characteristic of the nervous system can be used as a powerful tool to understand how the same behavior is regulated when the number of neurons varies by up to an order of magnitude (Tzouanas et al., 2019). Similarly, these animals can also be used to study neural regeneration following extensive damage (Gierer et al., 1972; Kücken et al., 2008; Miljkovic-Licina et al., 2007; Technau et al., 2000). These advantages make *Hydra* an excellent model organism to study behavior and circuit function in a highly plastic nervous system.

In addition to basic neuroscience and neural circuits, small animals and their compatibility with microscale technologies provide advantages for studies that benefit from high-throughput screenings. For example, *C. elegans* is a common model to study the protein aggregation related to diseases such as Alzheimer, Parkinson, Huntington, and ALS (Brignull et al., 2006; Harrington et al., 2011, 2010; Kaletta and Hengartner, 2006; Lublin and Link, 2013; Therrien and Parker, 2014). Similarly, zebrafish are commonly used to study epilepsy (Cho et al., 2017a; Eimon et al., 2018). Studies that use these model organisms have the potential to not only identify molecular pathways in disease pathogenesis but also screen for potential drug treatments and drug targets (van Ham et al., 2008; Kaletta and Hengartner, 2006; Mondal et al., 2016). Essential for these studies are readouts using behavior, electrophysiology, or fluorescence microscopy to quantify disease progression or drug efficacy. Although behavioral phenotypes combined with fluorescently tagged disease-related proteins are a common method to quantify protein aggregation, electrophysiological recordings offer a more detailed level of insight. Changes in electrical waveforms can indicate specific molecular pathways implicated in disease pathogenesis and also help identify targets for drug treatments. For example, nano-SPEAR electrodes embedded in a microfluidic device measured electrophysiology from *C. elegans* body-wall muscles and recorded distinct electrical phenotypes from ALS and Parkinson disease models (Figure 3D) (Gonzales et al., 2017). In addition, this technology found that drugs known to reduce protein aggregation in nematode Parkinson models also alleviated the electrophysiological phenotype (Figure 3D) (Gonzales et al., 2017). Similarly, using a scalable bioelectronic interface combined with drug screenings, researchers have used LFP measurements from the zebrafish central nervous system to identify potential anti-epileptic drug treatments (Eimon et al., 2018). Furthermore, bioelectronics and drug screenings have even been applied outside the domain of neurological disorders to deliver high-throughput screenings for treating infections due to parasitic nematodes (Figure 3E) (Lockery et al., 2012; Ménez et al., 2019; Weeks et al., 2016). As with applications to behavior and circuit dissection, conventional imaging techniques to monitor behavior or neural activity are powerful complementary methods to monitor the effects of drugs on animals, such as when the drug GSH is applied to induce feeding behavior in *Hydra* (Figure 3F).

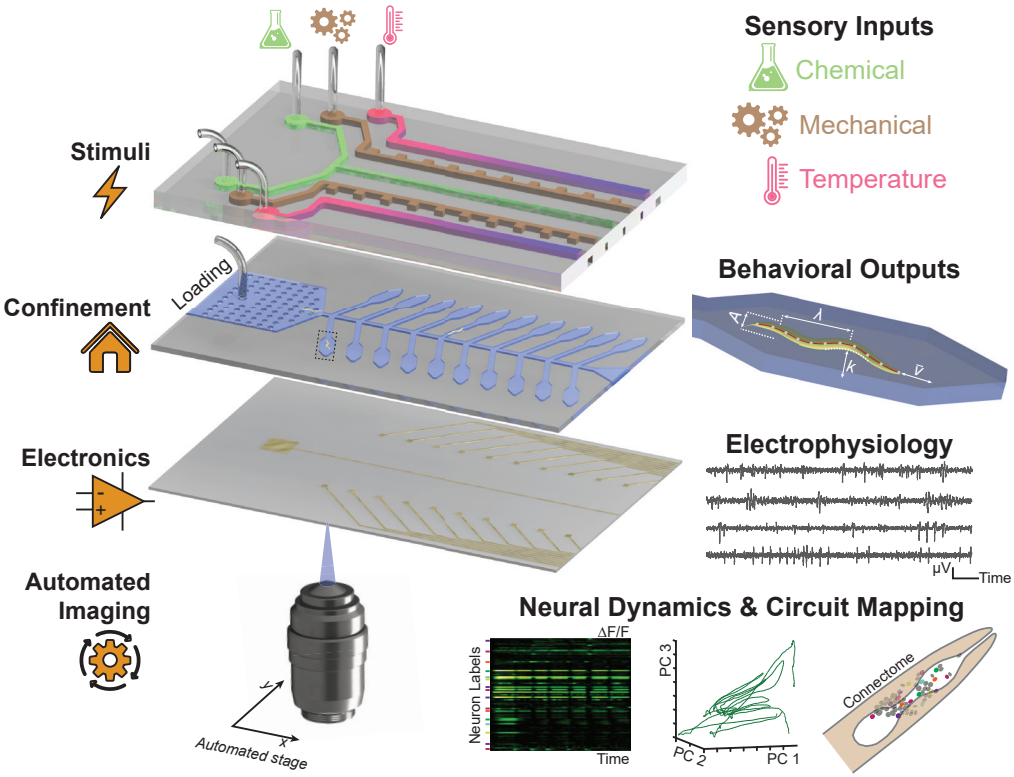

Figure 4. Multimodal Bioelectronic Platforms for Small Organisms

Future bioelectronic platforms will enable new discoveries by combining multiple cutting-edge technologies onto a single high-throughput platform. Electrophysiology and calcium-imaging data adapted from Gonzales et al. (2017) and Gonzales et al. (2019), respectively.

COMBINING BIOELECTRONICS WITH OTHER INTERROGATION MODALITIES

Although electrophysiological recordings give insight into the signaling of the nervous system, an advantage of millimeter scale bioelectronic devices is the combination of multiple experimental modalities typically not possible in larger mammalian animals. These include simultaneous functional calcium imaging, behavioral monitoring, and the ability to tightly control the microenvironment surrounding the animal (Figure 4).

Although electrophysiological and imaging modalities have already been discussed, these recordings can be combined with environmental manipulations such as chemical delivery, temperature regulation, and mechanical stimuli (Badhiwala et al., 2018; Cho and Sternberg, 2014; Han et al., 2017; Kopito and Levine, 2014; Larsch et al., 2015; Lockery et al., 2012; McClanahan et al., 2017; Nekimken et al., 2017; Swierczek et al., 2011; Tzouanas et al., 2019). For example, the substance reduced glutathione (GSH) can be delivered to *Hydra* with precise temporal control to induce mouth opening, which can be simultaneously imaged with GCaMP indicators (Figures 3C and 3F) (Badhiwala et al., 2018). Likewise, as previously mentioned, chemicals can be delivered in real-time to multiple nematodes during pharyngeal recordings to discover more potent anthelmintic drugs (Figure 3E) (Lockery et al., 2012; Weeks et al., 2016) or screen for drugs to treat diseases. In addition, food, odorants, and gasses can be tightly controlled in these devices to observe behavioral responses (Chronis et al., 2007; Gray et al., 2004; Kopito and Levine, 2014; Zimmer et al., 2009).

Microfluidic valves, typically used to control fluidic flow (Unger, 2000), can also be incorporated into bioelectronic devices for spatiotemporal control of mechanical stimuli (Cho et al., 2017b; Gonzales et al., 2019; McClanahan et al., 2017; Nekimken et al., 2017). The strength of these stimuli can be regulated by controlling the pressure used to rapidly inflate the valve. When combined with behavioral measurements or calcium imaging, devices can be scaled for high-throughput assays of *C. elegans* touch responses (McClanahan et al., 2017), revealing how mechanosensory neurons respond to touch (Cho and Sternberg, 2014; Nekimken et al., 2017), and used as a technique to test changes in sensorimotor responses during sleep (Gonzales et al., 2019).

Finally, as with chemical and mechanical stimuli, the environmental temperature can be temporally controlled. For example, raising the fluidic temperature increases *C. elegans* sleep behavior in microfluidic chambers (Gonzales et al., 2019). Moreover, rapid-flow microfluidic methods can be devised for controlling temperature in real time (Tzouanas et al., 2019). This technique recently revealed a *Hydra* behavioral response to temperature stimuli and indicated a potential thermally responsive circuit in the *Hydra* nervous system (Tzouanas et al., 2019).

Combining modalities for monitoring neural activity with the ability to precisely manipulate the environment allows researchers to study many aspects of neural circuits, behavior, and disease phenotypes (Figure 4). Furthermore, each of these tools can be tailored for specific organisms and incorporated onto a high-throughput, automated platform. This combined experimental substrate can not only yield large datasets across many conditions but also minimize variability through highly controlled environments and precise stimuli delivery (Figure 4).

FUTURE CHALLENGES

Bioelectronic devices for millimeter-sized animals have a number of advantages compared with conventional methods; however, there are still unique challenges to overcome moving forward. For example, current electrophysiological methods, such as nano-SPEARs, primarily record from muscle cells in animals such as *C. elegans* and *Hydra* (Gonzales et al., 2017). Despite the small form factor of these electrodes, they likely record the collective ensemble activity from several nearby cells. Although these electrophysiological recordings are physiologically and behaviorally relevant (Gonzales et al., 2017), a clear goal for the future of small-organism bioelectronics is to reliably record from many neurons with single-cell resolution and high signal-to-noise ratio. The ideal recording technique would likely involve nanoscale electrodes (AcarónLedesma et al., 2019), provide a significantly higher temporal resolution compared with calcium imaging, and mimic the resolution of gold-standard patch-clamp electrophysiology. A large body of work has demonstrated that it is possible to gain intracellular and quasi-intracellular (i.e., very high seal resistances) access to cells with nanoscale electrodes (Abbott et al., 2019, 2017; Angle et al., 2014; Duan et al., 2012; Fendyur and Spira, 2012; Jayant et al., 2019, 2017; Robinson et al., 2012; Tian et al., 2010; Vandersarl et al., 2012; Xie et al., 2012); however, implementing these interfaces to millimeter-sized animals requires surmounting significant challenges. Exquisite control of the animal and electrode placement would be required with a resolution of less than a micron. In addition, electrodes would need to be smaller than an individual cell, yet robust enough to penetrate the outer protective layers, which is no small feat for animals such as *C. elegans* that have tough outer cuticles. We envision a platform that reliably immobilizes animals (e.g. with microfluidic valves) and uses electroporation to gain *in vivo* and intracellular access. This technique would leverage electrode scalability for recordings. Even a small fraction (<10%) of electrodes strongly coupled to individual neurons or muscles would dramatically improve the scalability of patch-clamp electrophysiology.

In addition, there are inherent tradeoffs in combining multiple recording and stimulation modalities. In particular, both electrophysiology and calcium imaging often require some level of animal restraint (Badhiwala et al., 2018; Dupre and Yuste, 2017; Gonzales et al., 2019; Kato et al., 2015; Nichols et al., 2017; Vladimirov et al., 2018), even when state-of-the-art high-speed imaging techniques are used (Voleti et al., 2019). This confinement limits, and possibly even disrupts, normal animal behavior. A strategy to overcome this challenge is to study behaviors that occur despite animal confinement, such as microfluidic-induced sleep in *C. elegans* (Gonzales et al., 2017) and contractions in *Hydra* (Badhiwala et al., 2018; Tzouanas et al., 2019). When appropriate, alternative strategies such as virtual reality can also be used to simulate behavior, as is common in immobilized zebrafish during brain-wide imaging (Vladimirov et al., 2014). In addition, it is possible to fabricate chambers that allow for animal locomotion during electrophysiological recordings. For example, although *Hydra* are partially immobilized during nano-SPEAR recordings, animals still perform body contractions, which are measured as strong local-field potentials during recordings (see Figures 2B and 3C). Furthermore, on-chip chambers for *C. elegans* can be tailored to match the sinusoidal body posture of a crawling nematode (Gonzales et al., 2017). Nano-SPEAR recording electrodes embedded in the walls potentially allow for muscle cell recordings during semi-natural locomotion.

Another challenge is the order of magnitude difference in required imaging resolution when monitoring behavior or performing cellular-resolution calcium imaging. Behavior occurs on the millimeter scale, while neurons are only a few microns in diameter, therefore monitoring behavior and neural activity are often

done in separate experiments with dramatic differences in field of view and imaging resolution. To simultaneously record behavior and neural activity, multiple cameras can be employed during experiments (Nguyen et al., 2015; Venkatachalam et al., 2015), or wide-field calcium imaging with high spatial resolution can be used (Bouchard et al., 2015; Dupre and Yuste, 2017). However, this challenge is accentuated on high-throughput platforms when ideally many animals are monitored in parallel with a field of view spanning several centimeters. Although it is indeed possible to record behavior and single-neuron calcium activity across a population of moving *C. elegans* (Larsch et al., 2013), significant advances in wide-field microscopy will be necessary to scale these platforms to record from brain-wide activity in multiple moving animals simultaneously. We propose a strong alternative is to use high-throughput bioelectronic devices to confine many animals simultaneously (Figure 4). A wide-field camera can focus on a single animal for imaging both behavior and whole-brain activity during electrophysiology and external stimuli (Figure 4). When the experiment is completed, an automated stage moves the camera to image the next animal and an identical experiment is performed.

OUTLOOK AND PROSPECTS

We propose that bioelectronic devices for millimeter-sized animals can combine many techniques that are foundational to neurobiology onto a single high-throughput microdevice. Within these tiny experimental chambers many animals can be confined, multiple types of stimuli delivered as inputs to the animal, electrophysiology and calcium imaging used to monitor physiology and neural circuit activity, and behavioral outputs simultaneously recorded (Figures 2, 3, and 4). Although the nervous system of small model organisms show drastic anatomical differences compared with mammals, many molecular mechanisms related to development and signaling are conserved, and the large experimental throughput and percentage of the nervous system that can be simultaneously recorded provide advantages for researchers (Figure 1). In addition, many significant questions in neuroscience can be tackled with millimeter-sized animals regarding the fundamental input-output functions of neural circuits, the neural correlates of behavior, how brain-wide activity regulates behavioral state transitions (Figure 1). Furthermore, these animals are more tractable and scalable disease model alternatives to discover neuropathologies, discover drug targets, and search for therapeutics (Jones et al., 2005; Kaletta and Hengartner, 2006). Combining all of these aspects, we envision bioelectronics for millimeter-scale animals to be a field that continues to expand to provide fundamental discoveries and new tools for basic science.

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AUTHOR CONTRIBUTIONS

J.T.R. and D.L.G. outlined the manuscript. J.T.R., D.L.G., and K.N.B. wrote the manuscript. D.L.G., K.N.B., and B.W.A. prepared figures. All authors read and provided feedback on the manuscript. J.T.R. led the work.

DECLARATION OF INTERESTS

J.T.R, D.L.G., and B.W.A. are inventors on a patent for suspended nano-electrodes for on-chip electrophysiology filed by Rice University. All other authors declare no competing interests.

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