Review

Recent developments in VSD imaging of small neuronal networks

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Voltage-sensitive dye (VSD) imaging is a powerful technique that can provide, in single experiments, a large-scale view of network activity unobtainable with traditional sharp electrode recording methods. Here we review recent work using VSDs to study small networks and highlight several results from this approach. Topics covered include circuit mapping, network multifunctionality, the network basis of decision making, and the presence of variably participating neurons in networks. Analytical tools being developed and applied to large-scale VSD imaging data sets are discussed, and the future prospects for this exciting field are considered.

How do nervous systems produce behaviors? One fruitful approach to this question has been to focus on circuits containing relatively few neurons (e.g., 30–2000), usually in invertebrate ganglia, that produce well-characterized behaviors. Until recently, such networks were studied with sharp electrodes, limiting our view to the activity of two to four neurons at a time, but nonetheless allowing researchers to piece together circuit diagrams for various behaviors, including reflex withdrawals, feeding, and various escape responses. These networks have in turn been used to investigate mechanisms mediating higher-order processing such as decision making, pattern generation, and modulation (Getting 1989; Pittenger and Kandel 2003; Friesen and Kristan 2007; Marder and Bucher 2007; Briggman and Kristan 2008).

A watershed moment occurred with the development of voltage-sensitive dyes (VSDs) that allow neuronal activity to be recorded with light rather than with electrodes (Davila et al. 1973; Salzberg et al. 1973). Initially, this was accomplished by physically aligning multiple single photodiodes with selected neurons (Salzberg et al. 1977), but investigators soon began focusing the light from entire ganglia onto manufactured photodiode arrays (PDAs), opening the way for the simultaneous recording of dozens, and eventually, hundreds of neurons. Because PDAs achieve maximum signal-to-noise ratios at high light levels and high pass filter the signals before amplifying and digitizing them, they are typically used with fast absorbance VSDs to record action potentials rather than slower synaptic events or absolute membrane potential. The other type of recording platform in wide use, camera-based imaging systems, is used mainly with fluorescent VSDs to record both action potentials and synaptic potentials. The relative merits of these two recording platforms have been discussed elsewhere (Frost et al. 2010).

Much of the early work on VSDs was conducted by Larry Cohen and several others (Cohen 2010), who developed and screened large numbers of dyes to identify several having sufficient signal-to-noise ratios and low toxicity. One of the best of these, the absorbance dye RH155, was then used by their group to explore several issues in the marine mollusks *Navanax* (London et al. 1987) and *Aplysia* (Zecevic et al. 1989; Wu et al. 1994). One such study, which assessed the degree to which neurons in *Aplysia* are dedicated versus multifunctional with respect to different features of the animal's gill withdrawal reflex, concluded that both organizational schemes play a role. For example, although as expected many neurons responded to siphon stimulation, these were differentiable into a group whose firing correlated with reflex amplitude, another with reflex duration, and a third that correlated with both reflex components (Zochowski et al. 2000). Their finding was consistent with a prior realistic modeling study of the animal's siphon withdrawal reflex network constructed from sharp-electrode data (Lieb and Frost 1997), which suggested that some neurons and synaptic connections primarily mediate reflex amplitude, others reflex duration, and still others contribute to both.

Although Cohen subsequently shifted his attention to vertebrate preparations, several laboratories have since begun using VSDs to study small neuronal networks. Here we review recent work using VSDs to study such networks and highlight several of the advances that have been gained using this approach. Further we discuss tools being developed that can aid in extracting useful information from the enormous data sets produced by large-scale VSD imaging.

Leech segmental ganglia

Over four decades of research on the leech has led to a good understanding of the neuronal network distributed along the segmental ganglia that produce the animal's crawling and swimming behaviors (Friesen and Kristan 2007). During most of this time, this work was done with sharp electrodes, two to four neurons at a time. In 1999, William Kristan and colleagues introduced fluorescence resonance energy transfer (FRET) VSD imaging with a CCD camera to study this network (Cacciatore et al. 1999). This approach quickly led to the discovery of three neurons that were monosynaptic followers of a neuron known to halt swimming (Tr2). Two of these new cells, neurons 256 and 54, were then penetrated with intracellular electrodes and were found to stop the swimming motor program when stimulated, identifying them as intermediaries in the control of the leech swimming behavior (Taylor et al. 2003). This example of the use of VSD imaging

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demonstrates how it can speed the discovery of new neurons in known networks.

Kristan and colleagues subsequently used VSD imaging to explore the degree to which the leech swimming and crawling networks are organized according to a dedicated or multifunctional scheme (Briggman and Kristan 2006). Figure 1 shows their finding that 84 neurons in ganglion 10 are members of both networks. This large-scale imaging approach is consistent with findings obtained with traditional recording methods that many networks contain both dedicated and multifunctional neurons (Briggman and Kristan 2008).

Next, a landmark imaging study by Kristan and colleagues focused on the neuronal mechanisms underlying decision making (Briggman et al. 2005). Prior sharp-electrode studies had identified separate individual command neurons that can drive swimming versus crawling. Traditional thinking in the field considered such command neurons as the decision-makers for behavioral choices. However, the ability to record a large proportion of the total network provided an opportunity to test whether single neurons or groups of neurons mediate decision making. By treating the network as a dynamical system and applying principal component analysis followed by linear discriminant analysis, they identified a small group of neurons that collectively are the earliest predictors of what the animal will choose to do in response to a stimulus, firing even ahead of the command neurons. See below for further detail on this study, which exemplifies the power

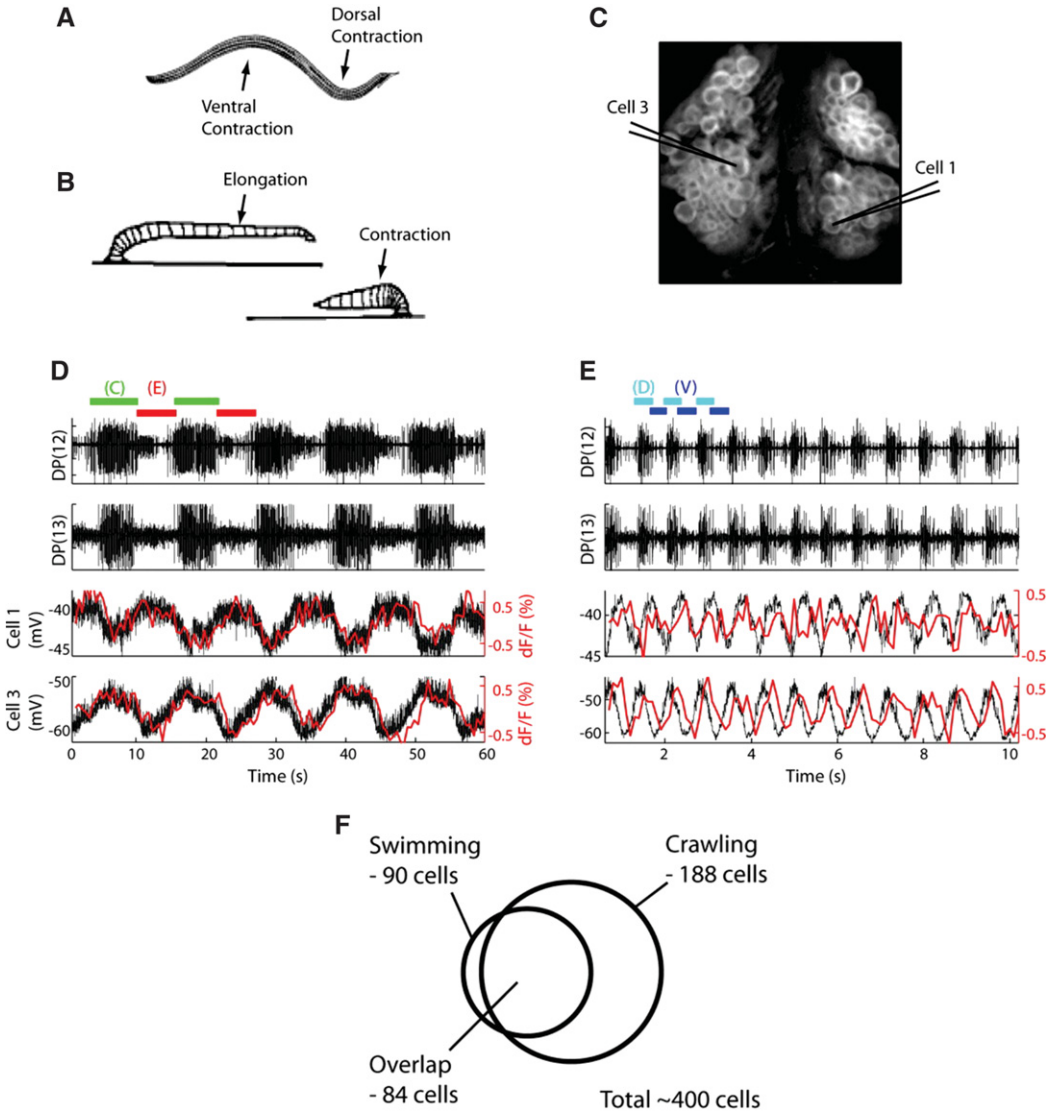

Figure 1. Imaging showed a high degree of overlap between the leech swimming and crawling networks. (*A*) The leech swimming behavior consists of alternating dorsal/ventral contractions of longitudinal muscles. (*B*) The crawling behavior is produced by contraction of circular muscles causing elongation followed by activation of longitudinal muscles causing contraction. (*C*) Image of the dorsal surface of ganglion 10, stained with a VSD. Cells 1 and 3 were impaled with sharp electrodes for simultaneous intracellular recording. (*D*) Simultaneous extracellular, intracellular, and optical recording during a crawling episode with alternating bouts of contraction (green bars) and elongation (red bars). Optical signals in cells 1 and 3 are shown in red overlaying the intracellularly recorded activity (black). Cells 1 and 3 oscillated 180° out of phase with each other during crawling. (*E*) Simultaneous extracellular, intracellular, and optical recording during a swimming episode with alternating dorsal/ventral flexions (*dorsal*—cyan bars, *ventral*—blue bars). Again, intracellular (black) and optical (red) signals for cells 1 and 3 are overlaid. Cells 1 and 3 oscillated 90° out of phase with each other during swimming. (*F*) Venn diagram showing the total number of cells in ganglion 10 that oscillated with swimming (90), with crawling (188), and the total number of cells that oscillated during both behaviors (84). (Adapted from Briggman and Kristan 2006 with permission from the Society for Neuroscience.)

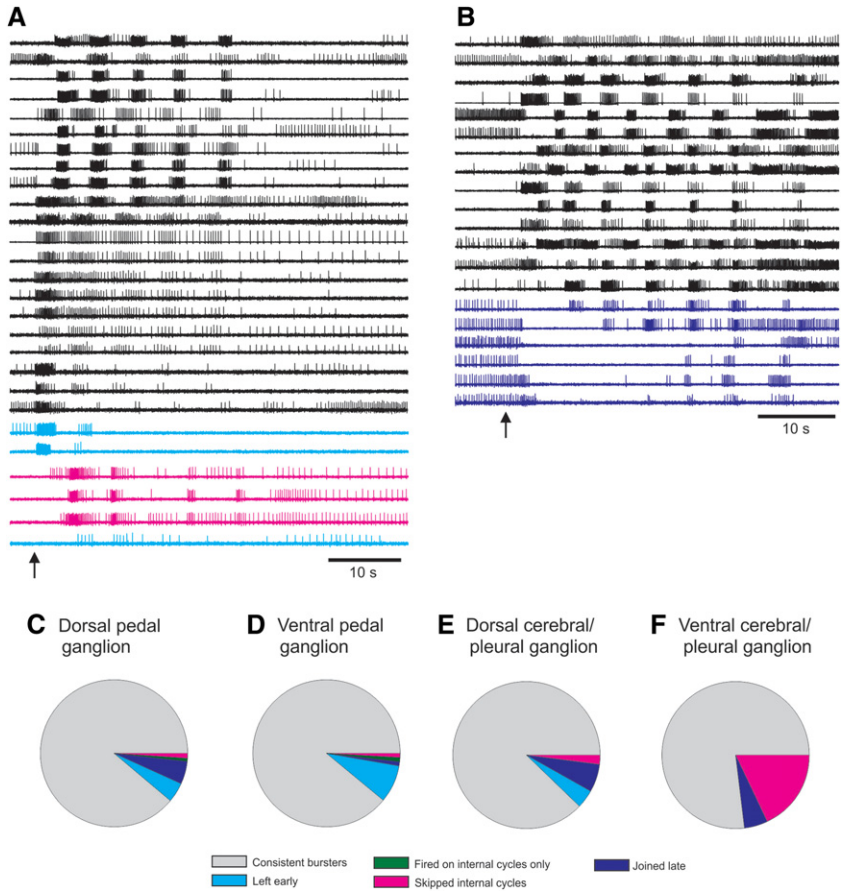

Figure 2. VSD imaging revealed that surprising network fluidity can underlie stereotyped motor programs. (*A*,*B*) Examples of optically recorded *Tritonia* SMPs in which some pedal ganglion neurons skipped cycles of the motor program. Note: The majority of pedal neurons fired bursts on every cycle of the motor program. (Arrows) Stimuli given to pedal nerve 3 to elicit motor programs. (*C*–*F*) Neurons that skipped cycles of the *Tritonia* SMP belonged to four categories: those that left early (cyan), fired on internal cycles only (green), skipped internal cycles (magenta), joined late (blue). Neurons that skipped cycles of the SMP were found in all *Tritonia* central ganglia (cerebral, pleural, and pedal).

of imaging combined with sophisticated analytical techniques to reveal higher-level cognitive features of network function.

Recently, Kristan and colleagues developed a new type of fast VSD that combines the best properties of electrochromic and FRET dyes (Miller et al. 2012). This new fluorescent dye uses a process known as photon-induced electron transfer, and has been shown capable of resolving action potentials in both rat hippocampal neurons and leech neurons (Miller et al. 2012). Continuing development of new VSDs with high signal-to-noise ratios and response speeds fast enough to resolve action potentials is important for the goal of imaging as many neurons as possible in the effort to understand how neuronal circuits produce behavior.

Tritonia escape swim network

In our laboratory we have adopted Cohen's method of using a fast absorbance VSD and a photodiode array, with which we are able to record action potential activity in up to 200 neurons simultaneously in central ganglia of the marine mollusks *Tritonia* and *Aplysia*. Raw data from the 464 diodes are spike-sorted into single neuron traces using independent component analysis (ICA) (Brown et al. 2001). ICA is a fully automated, blind source separation procedure that transforms data so that the resulting independent components are maximally statistically independent. We recently confirmed the accuracy of ICA spike-sorting in experiments where intracellular and optical recording were performed together. In all cases, each intracellular trace was found to match, spike-for-spike, one of the ICA spike-sorted traces (Hill et al. 2010).

Using this approach, we quickly made the unexpected discovery that many neurons participate quite variably in otherwise stereotypic, rhythmic motor programs in three molluscan species. Figure 2, A and B, shows examples of *Tritonia* pedal ganglion neurons skipping cycles of the escape swim motor program (SMP). Such variably participating neurons were found in the cerebral, pleural, and pedal ganglia of *Tritonia* (Fig. 2C–F). Further, these loosely committed neurons varied their level of participation from motor program episode to episode (Hill et al. 2012b). This finding is consistent with studies in vertebrates that report trial-to-trial variability in neurons participating in what appear to be unvarying behaviors (Carmena et al. 2005; Ziv et al. 2013). Taken together, these studies support the idea that networks, even for very stereotypic behaviors, may be more fluid in their moment-to-moment functional structure than generally envisioned.

As in the leech, VSD imaging has proven useful for identifying new neurons in the *Tritonia* swim network (Frost and Wu 2014). One tool that has facilitated this is our construction of a hybrid microscope having two lens systems, one for imaging and another for providing stereopsis for depth perception when using sharp electrodes to penetrate neurons of interest identified by optical recording (Frost et al. 2007). The ability of large-scale imaging to accelerate circuit mapping has the potential to stimulate comparative studies of neuronal networks, which, as emphasized in a recent NSF workshop, are key routes to the discovery of general principles of brain function (Striedter et al. 2014).

Aplysia locomotion network

Aversive tail stimuli elicit escape crawling in *Aplysia*, which is driven by a rhythmic series of muscular contraction cycles that travel repeatedly head-to-tail down the length of the animal, propelling it forward. Sharp electrode studies of the network mediating this behavior have identified a large number of neurons in the pedal ganglion that burst with each muscle contraction cycle; some of these have been identified as motor neurons, with others suggested to be modulatory neurons (Jahan-Parwar and Fredman 1978; Hening et al. 1979; Fredman and Jahan-Parwar 1980, 1983; McPherson and Blankenship 1991; Romanova et al. 2007). However, little is known about the functional organization of these neurons. Our recent imaging studies reveal that during the crawling motor program, firing progresses through a series of neuronal ensembles in a continuous, repeating manner (Fig. 3A; AM Bruno,

WN Frost, MD Humphries, in prep.), presumably driving the orderly sequence of muscle contractions that pass head-to-tail down the animal on each cycle of the motor program.

Our imaging work in *Aplysia* has also reinforced some of the findings described above from *Tritonia*. First, combined optical and sharp-electrode recordings in the *Aplysia* buccal ganglion have confirmed the accuracy of automated ICA spike-sorting of raw PDA data into single neuron traces (Hill et al. 2012a). Second, optical recordings of the *Aplysia* locomotion motor program revealed the presence of variably participating neurons that burst on some but not all cycles, extending the generality of this finding (Hill et al. 2012b).

Crustacean stomatogastric ganglion

The crustacean stomatogastric ganglion (STG) is a small neuronal network consisting of some 30 cells, which produces chewing and filtering behaviors. Decades of work with sharp electrodes has provided a detailed understanding of the connectivity between STG neurons, as well as the numerous modulators that affect different synapses among STG neurons (Marder and Bucher 2007). Fluorescent VSD imaging has recently been used to image the activity of neurons in both the lobster and crab STG (Stadele et al. 2013). The authors showed that they could resolve action potentials as well as excitatory and inhibitory synaptic potentials, and could stably image neuronal activity for many hours. The ability to record synaptic potentials from many STG neurons for extended periods offers a great opportunity to examine the long-term effects of neuromodulators on subthreshold potentials, a difficult feat with sharp electrodes due to electrode drift (Stadele et al. 2013). Given its potential for recording all STG neurons simultaneously, VSD imaging may significantly extend our understanding of how this small network operates.

Vertebrate respiratory networks

VSD imaging, although without single-neuron resolution, has also recently been applied to the vertebrate pre-Bötzinger complex and other areas of the ventral medulla involved in respiration. The pre-Bötzinger complex contains ~1000 neurons, and plays a key role in the genesis of the respiratory rhythm (Feldman et al. 2013). The neurons of the pre-Bötzinger complex drive inspiration via projections to neurons in premotor areas of the medulla, which in turn drive inspiratory muscles such as the diaphragm and the external intercostals (Feldman et al. 2013). Recently, it has been shown that stimulation of only three to nine inspiratory

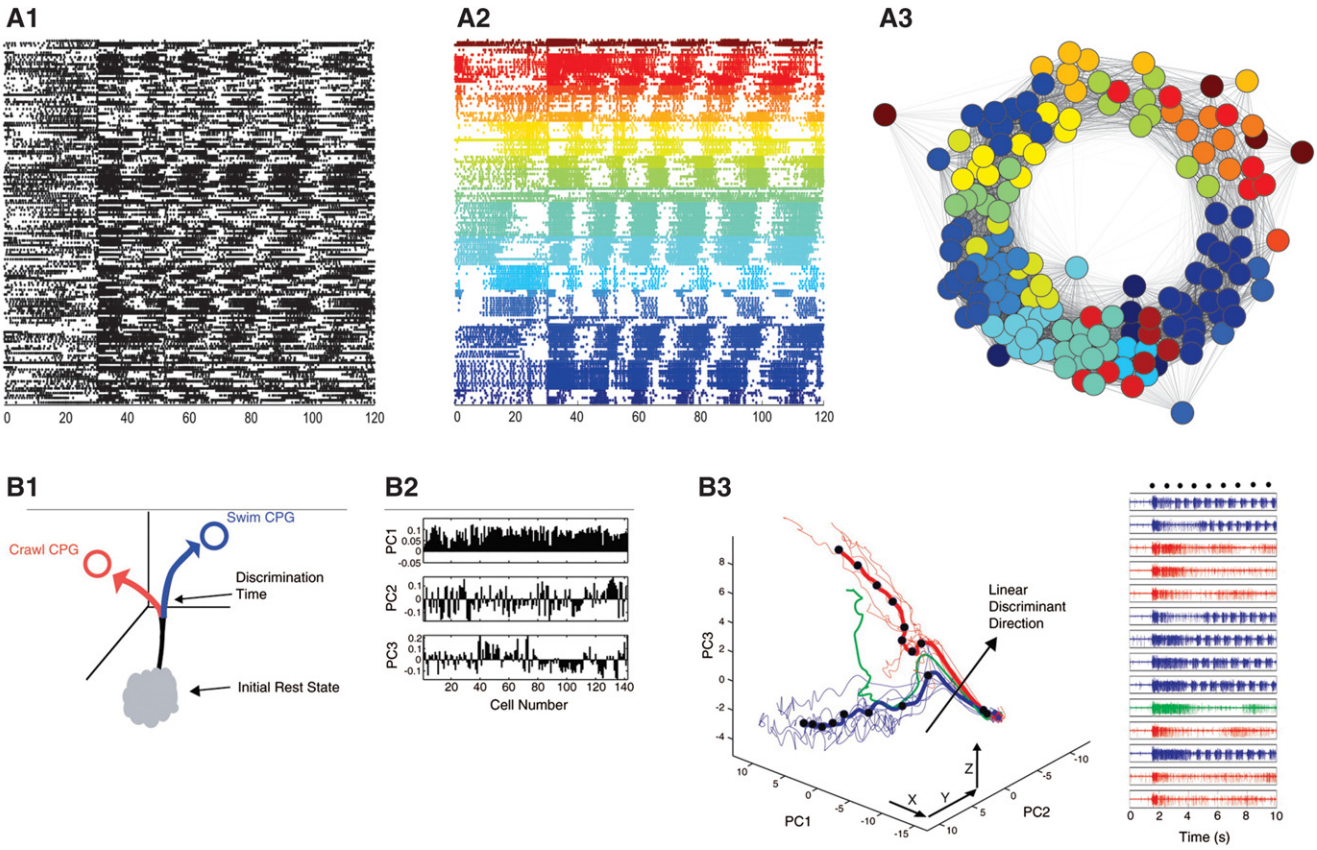

Figure 3. Examples of analytic tools used in small network imaging studies. (*A*) Use of graph theoretic-based clustering to derive the ensemble organization of the *Aplysia* locomotion motor program. (*A1*) Action potential recording of 155 neurons. At 30 sec into the 2-min recording, a nerve stimulus was applied to trigger the rhythmic locomotion motor program. (*A2*) Unsupervised consensus clustering yielded 22 significant ensembles by their correlated firing. (*A3*) Visualization of the degree of correlation of all recorded neurons, revealing the order of firing of the ensembles. Distance between neurons indicates their degree of correlated firing. The ensembles clearly fire in an orderly, repeating sequence. (*B*) Use of PCA and LDA to study decision-making in the leech. (*B1*) Abstract representation of behavioral state as a trajectory phase space following a stimulus. The divergence point would represent a time at or near the decision point. (*B2*) Contributions of all 143 recorded neurons to each of the first three principal components. (*B3*) Trajectories of a 14 trial experiment. *Right* panel shows a nerve recording from each trial. The same stimulus led to a decision to swim in the blue trials, and to crawl in the red trials. The green trial was one in which the brain changed its mind, and switched from a swimming to a crawling motor program in midstream. (Adapted from Briggman et al. 2005 with permission from the American Association for the Advancement of Science.)

phase neurons can initiate respiration network activity, which then rapidly "percolates" through the network (Kam et al. 2013). Researchers have begun using VSD imaging to gain insights into how this small network operates. For example, imaging of the pre-Bötzinger complex has shown that areas not active during eupnea are recruited with gasping (Potts and Paton 2006). Further, VSD imaging revealed that an area of the ventral medulla localized ventrolateral to the facial nucleus (the pFRG) shows pre-inspiratory population activity. Lesioning of this area leads to a reduction in respiratory frequency and to a change in the spatiotemporal pattern of respiratory neuron activity, suggesting that the pFRG is involved with respiratory rhythm generation (Onimaru and Homma 2003). Thus, VSD imaging has led to the identification an area of the ventral medulla involved in respiratory rhythm generation and has also revealed how network activity in the pre-Bötzinger complex is altered in different circumstances. These findings illustrate the usefulness of VSD imaging for efforts to understand how networks in the mammalian ventral medulla produce respiratory rhythms.

Vertebrate enteric ganglia

The mammalian enteric nervous system (ENS) is a self-contained network of neurons that produces the complex behaviors that together comprise normal gastrointestinal function. ENS neurons range in size from 10 to 25 microns and are arranged in plexuses located in the wall of the gut. The myenteric plexus is located between the longitudinal and circular muscle layers, and the submucosal plexus is located between the circular muscle layer and the mucosa. Since the ENS plexuses are organized essentially in two dimensions, they are particularly attractive for optical imaging studies. In fact, the first optical recordings of electrical activity in the mammalian nervous system with single-cell resolution were performed in the submucosal plexus of the guinea-pig small intestine (Obaid et al. 1999). In that study, the authors found that nicotinic acetylcholine receptors (nAChRs) that reversibly desensitize following nicotine exposure may be responsible for the enhancement of neuronal activity observed following nicotine application (Obaid et al. 1999). The same group then used VSD imaging to show that nAChRs may be capable of regulating the activity of both excitatory and inhibitory pathways (Obaid et al. 2005). Similarly, another group used VSD imaging to examine the activity of neurons in the guinea-pig and mouse ENS (Neunlist et al. 1999), and have gone on to use this technique to record the activity of cultured human myenteric neurons, recording spike discharge following nicotine application (Vignali et al. 2010). VSD imaging, with its ability to record the activity of many neurons simultaneously in the two-dimensionally arranged ENS, promises to further increase our understanding of how this complex, distributed network of neurons functions to produce healthy gastrointestinal function.

Special analysis tools are needed to mine the data obtained from large-scale imaging

Optical recording, with its ability to simultaneously record large numbers of neurons, is ideal for exploring network-level issues such as how circuits reorganize to store memory, or whether decision making involves top down or more consensus-like mechanisms. Although some topics can be addressed through simple visual inspection of the data, in most cases more sophisticated approaches are necessary. As many have noted, the next frontier in network neuroscience depends on the development and use of analytical methods for revealing signatures of network organization and the corresponding computations performed by those network structures (Briggman et al. 2006; Koch 2012; Alivisatos et al. 2013; O'Leary and Marder 2014).

For example, learning studies can benefit from methods for tracking changes in the network ensemble structure with experience. Many methods have been developed to partition data sets into ensembles of neurons having significantly correlated firing patterns (Feldt et al. 2009; Humphries 2011; Lopes-dos-Santos et al. 2011; Lyttle and Fellous 2011; Gerstein et al. 2012). Our laboratory has used an unsupervised graph theoretic-based clustering approach to reveal the existence of physically segregated ensembles of neurons that are re-identifiable across preparations during the *Aplysia* locomotion motor program (Fig. 3A; Bruno et al. 2013; AM Bruno, WN Frost, MD Humphries, in prep.). These can then be tracked across training trials to identify ensembles involved in learning and to characterize the changes in network organization that encode memory. The same approach could be used to investigate how networks functionally reorganize in response to modulation, injury, and disease.

More complex analysis tools are needed to investigate higher-order features of network function, such as those related to cognitive processing. For example, are behavioral decisions based on top-down or consensus-like, deliberative network mechanisms? As described earlier, Kristan and colleagues (Briggman et al. 2005) combined PCA with linear discriminant analysis (LDA) to test whether single neurons or groups of neurons mediate decision making in the leech. PCA was used as a first step to reduce the dimensionality of the data set, which helped distinguish between the crawling versus swimming motor programs and visualize the divergence point associated with the brain's decision regarding which behavior to choose (Fig. 3B). Such PCA-based dynamical portraits are increasingly used to provide useful insights into computations performed by both vertebrate and invertebrate networks (Niessing and Friedrich 2010; Churchland et al. 2012). The leech study then used LDA to identify the time point when the trajectories for swimming and crawling significantly diverged. After finding that decision making was correlated with activity in a defined group of neurons, they then used sharp electrodes to find one that acted to bias the decision to swim or crawl. Their work implicated that the earliest moment of decision making involves consensus-like correlated activity in a group of neurons, which occurs earlier than the activity in the previously identified individual command-type neurons in this system (Kristan 2008).

Logistic regression, which is similar to LDA, has been applied to multi-electrode array data from primate cortex by Newsome and colleagues to discriminate the neural correlate of changes of mind during decision making in primates (Kiani et al. 2014). The study involved the simultaneous recording of nearly 200 units from a circuit containing millions of neurons. This work was not concerned with identifying the contributions of individual neurons, but rather with the deliberative process of the recorded sub-network during changes of mind. Interestingly, in the leech study discussed above, a subset of trials found that the much simpler invertebrate preparation could also start to make a decision and then subsequently change to a different behavioral choice, raising the opportunity to explore whether changes in mind during decision making may involve similar underlying principles in simple and complex brains.

Although it is beyond the scope of this review to cover the array of analytical tools available (see Briggman et al. 2006), the few studies highlighted here show how their application to large-scale recordings can expose fundamental features of cognitive processing and predict behavioral outcomes. The future of brain network analysis depends critically on the development and application of such tools (Alivisatos et al. 2013). Their use in a variety of preparations, from vertebrate to invertebrate, will be key to uncovering general principles of network function.

What lies ahead for VSD imaging of small neuronal networks?

Small neuronal networks remain attractive model systems for discovering general principles of nervous system function (Selverston 1999; Pittenger and Kandel 2003; Clarac and Pearlstein 2007; Katz et al. 2013). Such networks offer the advantage that a significant fraction of the total network can be recorded simultaneously. Further, the behavioral relevance of specific neurons and ensemble types can be evaluated. These features also make small networks useful for bench-testing analysis tools that can be applied equally well to vertebrate networks (Humphries 2013; AM Bruno, WN Frost, MD Humphries, in prep.).

Although many of the imaging findings reviewed here confirm insights already gained from sharp-electrode studies, optical recording greatly increases the speed with which such advances can be made. For example, a single imaging experiment can reveal the basic organizational features of a given network, e.g., how many neurons and ensemble types participate in a motor program (Fig. 3A), orders of magnitude faster than can be accomplished with sharp electrodes. As mentioned earlier, this may encourage comparative studies, which are necessary for establishing whether given phenomena are general features of neuronal networks.

Given the rapidly expanding array of large-scale recording methods and analysis tools, this is an exciting time in systems neuroscience. In addition to the topics reviewed here, several areas are increasingly open for exploration. For example, what organizing features of network function are common across species? How flexible are network structures moment-to-moment and across longer intervals of time? What is the role of spontaneous activity in determining how inputs are interpreted and processed? How do damaged networks regain function after injury? Small, typically invertebrate, neuronal networks remain ideal preparations in which to explore such fundamental issues of brain function.

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